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Resilience to social defeat-induced increased in ethanol intake: neuroinflammation response

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CERTIFICAN

Que la Tesis Doctoral presentada por Doña Marina Daiana Reguilón Romero, con el título "Resilience to social defeat-induced increased in ethanol intake: neuroinflammation response" ha sido realizada bajo su dirección. Tras haberla examinado hacen constar su autorización para que se realicen los trámites conducentes a su defensa.

Y para que conste a los efectos oportunos, firman el presente certificado en Valencia a 15 de septiembre de 2022.

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A mi madre, Angélica

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PREFACE

This Doctoral Thesis is based on the following seven studies:

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- Reguilón, M. D., Ferrer-Pérez, C., Ballestín, R., Miñarro, J., & Rodríguez-Arias, M. (2020). Voluntary wheel running protects against the increase in ethanol consumption induced by social stress in mice. *Drug and alcohol dependence, 212*, 108004. https://doi.org/10.1016/j.drugalcdep.2020.108004 **(Study 2)**
- Reguilón, M. D., Ferrer-Pérez, C., Manzanedo, C., Miñarro, J., & Rodríguez-Arias, M. (2021). Ethanol intake in male mice exposed to social defeat: Environmental enrichment potentiates resilience. *Neurobiology of stress, 15*, 100413. https://doi.org/10.1016/j.ynstr.2021.100413 **(Study 3)**
- Reguilón, M. D., Miñarro, J., & Rodríguez-Arias, M. Voluntary exercise pre-exposure during adolescence enhances resilience in rodents subjected to social defeat and ethanol self-administration in adulthood. *In preparation.* **(Study 4)**
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- Reguilón, M.D. Morphological changes induced by social stress and ethanol intake in male mice. *Pilot study.* **(Study 7)**

Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropic Hormone
APC	Antigen-presenting cells
AUD	Alcohol Use Disorder
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BNST	Bed Nucleus of the Stria Terminalis
CA1	Cornu Ammonis 1
CeA	The Central Amygdala
CNS	Central Nervious System
CPP	Conditioned Place Preference
CRH	Corticotropin Releasing Hormone
DA	Dopamine
DAMP	Damage-Associated Molecular Patterns
DID	Drinking in the Dark
EPM	Elevated Plus Maze
EE	Environmental Enrichment
FR1	Fixed Ratio 1
FR3	Fixed Ratio 3
GC	Glucocorticoids
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptor
HPA	Hypothalamic–Pituitary–Adrenal Axis
IFN	Interferons
IL	Interleukin

KO	Knockout
LC	Locus Coeruleus
MAP-2	Microtubule-Associated Protein 2
NAc	Nucleus Accumbens
NE	Norepinephrine
NF200	Neuronal Filaments 200 kDa
NPY	Neuropeptide Y
OXT	Oxytocin
PAMP	Pathogen-Associated Molecular Pattern
PFC	Prefrontal Cortex
PND	Postnatal Day
PR	Progressive Ratio
PrL	Prelimbic Cortex
PRR	Pattern Recognition Receptor
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus
S-100β	S100 Calcium-Binding Protein β
SA	Self-Administration
SD	Social Defeat
SIT	Social Interaction Test
SUD	Substance Use Disorder
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
VTA	Ventral Tegmental Area

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RESUMEN AMPLIO

INTRODUCCIÓN

El estrés puede definirse como una respuesta biológica inespecífica del organismo a una demanda formulada desde el exterior (el entorno). Se trata de una respuesta normal y adaptativa, en la que se movilizan las reservas del organismo, se facilita la captación de energía por parte de la musculatura, se eleva el tono cardiovascular para transportar oxígeno más rápidamente y se suspenden las actividades no esenciales (Selye, 1975a, 1975b). A pesar de que el estrés es una respuesta normal y adaptativa, cuando es de gran intensidad o de carácter crónico, se producen marcados efectos en el organismo que dan lugar a diferentes tipos de trastornos (Sandi & Haller, 2015; Selye, 1975a, 1975b). Los conflictos sociales se producen con frecuencia tanto en la sociedad humana como en el mundo animal, provocando un estrés inevitable que empeora la calidad de vida induciendo diferentes enfermedades como la ansiedad, la depresión, el trastorno por estrés postraumático (TEPT) o la vulnerabilidad a desarrollar un trastorno por uso de sustancias (TUS; Daviu et al., 2019; Sandi & Haller, 2015; Vasconcelos et al., 2015).

La respuesta biológica al estrés permite al organismo actuar de forma rápida y eficaz con su entorno. Si la situación estresante es transitoria, los mecanismos biológicos innatos permiten al organismo adaptarse a las circunstancias y dar una respuesta adecuada para resolver la situación. Si la situación estresante se vuelve crónica, la propia respuesta biológica del organismo acaba siendo perjudicial para el mismo (Sapolsky, 1996; Lijffijt et al., 2014). Diferentes sistemas fisiológicos del organismo se activan con el fin de adaptarlo a los retos internos o externos. Entre ellos, cabe destacar el eje hipotálamo-hipofisario-adrenal (HHA), un sistema que ejerce un papel fundamental a través de la hormona liberadora de corticotropina (CRH), la hormona adrenocorticotrópica (ACTH) y los glucocorticoides (GC). También cabe destacar el sistema simpático suprarrenal, con la estimulación de la producción y

liberación de adrenalina y noradrenalina (NA; Kupfermann, 1991). Aun así, hay otros sistemas que se activan en la respuesta al estrés, como, por ejemplo, el sistema inmunitario (véase la revisión de Capuron & Miller, 2011; Costa-Pinto & Palermo-Neto, 2010).

Una de las principales fuentes de estrés es el llamado estrés social. Las interacciones sociales son esenciales en el desarrollo de los seres humanos y de la mayoría de las especies animales. Sin embargo, algunos eventos sociales pueden provocar efectos adversos como la depresión y la ansiedad, e incluso pueden influir en el consumo de drogas de abuso (Cadet, 2016; Cohen et al., 2016). Los estresores emocionales y sociales son los principales activadores de la respuesta al estrés en humanos, lo que explica la importancia traslacional del estudio del estrés social. Los modelos animales pueden arrojar luz sobre los mecanismos neurobiológicos que subyacen a los efectos del estrés social y que serían imposibles de investigar de otro modo. La investigación preclínica lleva décadas estudiando los mecanismos implicados en la respuesta al estrés y el desarrollo de trastornos mentales asociados a éste. En este sentido, se han desarrollado varios paradigmas que emulan el estrés social en roedores, como la derrota social, la privación social, la inestabilidad social y la agresión territorial y materna. La derrota social se considera el paradigma más representativo para estudiar las consecuencias fisiológicas y conductuales del estrés social. La derrota social induce numerosos cambios conductuales, de los cuales los más estudiados son la disminución de la interacción social (Montagud-Romero et al., 2018; Toth & Neumann, 2013) o el aumento de la anhedonia (Heshmati & Russo, 2015; Riga et al., 2015; Shimamoto et al., 2015), que pueden extrapolarse e interpretarse como indicadores de ciertas patologías observadas en humanos, como la depresión. En diferentes estudios preclínicos se ha observado que los animales derrotados en la edad adulta pueden presentar alteraciones en el aprendizaje espacial, aumento de la ansiedad y alteración del comportamiento social (Blanco-Gandía et al., 2019; García-Pardo et al., 2015). Los efectos del estrés físico o farmacológico

sobre la plasticidad sináptica han sido ampliamente estudiados, encontrando que inducen una reducción de la proliferación celular y un aumento de la muerte celular, disminuyen la densidad sináptica y reducen el volumen del hipocampo, ya sea cuando se experimenta durante la adolescencia o la edad adulta (ver revisión Bath et al., 2017; Montes-Rodríguez & Urteaga-Urias, 2018). Los cambios en la morfología dendrítica y del citoesqueleto axonal inducidos por la derrota social han sido menos estudiados, aunque hay indicios de que el estrés social, a través de diferentes modelos (derrota social, estrés crónico leve imprevisible y separación maternal), produce alteraciones en las extensiones dendríticas y alteración sináptica (Abdel-Rahman et al, 2004; Barros et al., 2006; García-Gutiérrez et al., 2016; Martin et al., 2017; Yang et al., 2015; García-Gutiérrez et al., 2016). Además, la derrota social induce cambios estructurales en regiones cerebrales clave en la conducta de recompensa, adaptaciones que inducen la vulnerabilidad a la adicción. Recientemente, se ha observado que hay una reducción de la transmisión sináptica inhibitoria dentro del núcleo accumbens (NAc) que es crítica en la respuesta al estrés (Heshmati et al., 2020).

La adicción es una enfermedad crónica y multifactorial que se caracteriza por un comportamiento fuera de control y una recaída en el consumo, incluso después de un período prolongado de abstinencia (Koob & Volkow, 2016). Entre los principales factores de riesgo implicados en el TUS, el estrés es uno de los más importantes. El estrés no sólo juega un papel fundamental en la recaída en el consumo de drogas, sino también en el inicio, escalada y mantenimiento del patrón de consumo. Las experiencias estresantes modifican la actividad de las áreas cerebrales implicadas en los efectos gratificantes de las drogas (Beutel et al., 2018; Ferrer-Pérez et al., 2018a, 2018b; García-Pardo et al., 2015; Hwa et al., 2016; Montagud-Romero et al., 2021; Newman et al., 2018; Rodríguez-Arias et al., 2013, 2016, 2017). La exposición al estrés agudo produce un aumento de GC que induce la actividad de la dopamina (DA) en el área tegmental ventral (ATV), pero cuando el estrés se vuelve crónico se

produce una circulación elevada de GC y una supresión selectiva de la capacidad del CRH para modular la liberación de DA en el NAc (Douma & de Kloet, 2020). Así, el estrés crónico induce una disminución de la liberación de DA en las regiones límbicas y corticales (Douma & de Kloet, 2020), con la consiguiente disminución de las sensaciones placenteras (Tye et al., 2013).

El alcohol es la sustancia de abuso más consumida en todo el mundo (EDADES, 2019-20; OMS, 2018). Las consecuencias del consumo abusivo, crónico e incluso de los atracones de alcohol son diversas y dan lugar a una amplia gama de enfermedades (cardiovasculares, hepáticas, déficits cognitivos, etc.; Nutt et al., 2010). Estudios preclínicos han demostrado que la exposición a derrota social induce un aumento del consumo voluntario y de la motivación para obtener una dosis de esta sustancia en roedores adolescentes y adultos (Favoretto et al., 2020; Karlsson et al., 2017; Macedo et al., 2018; Norman et al., 2015; Rodríguez-Arias et al., 2016). Además, hay que tener en cuenta que el alcohol se considera una "puerta de entrada" al consumo de otras sustancias de abuso y es una de las drogas de inicio más temprano, junto con el tabaco y los hipnosedantes (EDADES, 2019-20). Por ello, el estudio de los efectos inducidos por el estrés sobre el consumo de sustancias de abuso, como el alcohol en los jóvenes adolescentes es un tema de gran interés. La adolescencia es un periodo crítico de maduración cerebral caracterizado por múltiples cambios cognitivos, conductuales y biológicos relacionados con importantes cambios en las funciones emocionales y cognitivas (Pickles et al., 1998, Spear, 2000; Bava & Taper, 2010). Este periodo se caracteriza por comportamientos comunes a todas las especies, como la hiperfagia, el retraso del ciclo circadiano, el aumento de las interacciones sociales, la mayor búsqueda de novedades y los comportamientos de riesgo (Spear, 2000). La activación de las hormonas esteroideas durante la adolescencia desencadena numerosos efectos organizativos, dando lugar a cambios estructurales, como la mielinización, la apoptosis, la poda neuronal y la remodelación de las espinas dendríticas, determinando así la respuesta conductual

adulta a los esteroides o a los estímulos sensoriales (Vigil et al., 2011). La maduración cerebral en la adolescencia afecta a todo el cerebro, en el que se produce una importante pérdida de materia gris y del número de sinapsis, y de receptores sinápticos principalmente en el neocórtex, así como, un aumento de la materia blanca relacionado con el incremento de la mielinización de las conexiones interhemisféricas y cortico-subcorticales (Gogtay et al., 2004). La adquisición de habilidades cognitivas maduras en los dominios de la toma de decisiones, la inhibición del comportamiento y la memoria de trabajo se atribuye a la maduración funcional de la corteza prefrontal durante la adolescencia. Sin embargo, durante la adolescencia se experimenta una disminución de los principales receptores de neurotransmisores. Principalmente se produce una poda sináptica excitatoria, es decir, hay una disminución de los receptores NMDA y de la extensión de la estimulación glutaminérgica excitatoria en el córtex. Por el contrario, la inhibición GABAérgica es mayor durante la adolescencia debido a los cambios pre y postsinápticos, y el número de sinapsis GABA aumenta (Caballero et al., 2016; Zimmermann et al., 2019). En las regiones límbicas también hay cambios importantes, con reducciones de la materia gris en el hipocampo, el estriado y otras estructuras subcorticales, lo que conlleva aumentos de la materia blanca en los tractos de fibras corticales y subcorticales, y en las conexiones entre la amígdala y el estriado con la corteza prefrontal (Caballero et al., 2016; Zimmermann et al., 2019). Además, hay una disminución de los receptores de glutamato en el hipocampo y de los receptores DA en el estriado (Zimmermann et al., 2019). La corteza prefrontal requiere más tiempo para alcanzar la madurez en comparación con las regiones límbicas durante la adolescencia, lo que resulta en una falta de sincronía entre el área frontal y las regiones cortico-subcorticales. Esto conduce a una elevada activación en las regiones mesocorticolímbicas implicadas en la conducta de refuerzo y a una atenuación de la sensibilidad a los estímulos aversivos, lo que da lugar a una conducta de búsqueda de novedades con conductas de riesgo y una mayor

reactividad emocional (Sturman & Moghaddan, 2011). También es un periodo especialmente vulnerable a las consecuencias negativas del estrés y asociado al inicio del consumo de drogas como el alcohol (Burke & Miczek, 2014; Spear & Swartzwelder, 2014).

Así, la adolescencia se caracteriza por la disminución de la capacidad de autorregulación como consecuencia de la limitada capacidad inhibitoria, la escasa regulación del control, la hiperactividad de la amígdala y la hiperactividad de la DA en el NAc al procesar los estímulos apetitivos (Rodríguez-Arias & Aguilar, 2012). Además, la adolescencia se considera una época especialmente estresante debido a los grandes cambios que se producen, y es muy común que los individuos perciban las situaciones estresantes con mayor intensidad y puedan sentirse sobrecargados. Cuando esto ocurre, el estrés mal gestionado puede conducir a la inestabilidad emocional, la ansiedad, el retraimiento, la agresividad, las enfermedades físicas o las habilidades de afrontamiento inadecuadas, como el uso de drogas como el alcohol (Buchanan et al., 1992). A menudo se ha descrito que la sensibilidad conductual a las recompensas alcanza su punto máximo durante la adolescencia y luego disminuye gradualmente durante la edad adulta (Steinberg et al., 2009; Steinberg, 2010). La sensibilidad a una recompensa básica es mayor durante la adolescencia temprana (Desor & Beauchamp, 1987; Doremus-Fitzwater et al., 2010). Durante esta etapa hay una disminución temporal de la eficacia de las proyecciones mesolímbicas de DA, hay una menor tasa basal de liberación de DA con una mayor captación y menor liberación de DA en comparación con los adultos (Stamford, 1989). Esta menor activación llevaría a los adolescentes a buscar mayores sensaciones y recompensas y a realizar conductas más arriesgadas en un intento de compensar el déficit dopaminérgico. La sensibilidad a la recompensa y la elevada asunción de riesgos que experimentan los adolescentes fomenta la experimentación temprana con drogas porque, en general, son más gratificantes y menos aversivas (Schramm-Sapyta et al., 2009). La exposición y el consumo de drogas durante la adolescencia suelen predecir

una mayor probabilidad de consumo continuado en la edad adulta (Merline et al., 2004; Spear, 2015). El alcohol es la droga de primera elección entre los adolescentes. Los estudios clínicos y preclínicos confirman que el consumo excesivo de alcohol durante este periodo sensibiliza regiones cerebrales y procesos de desarrollo que están implicados en las conductas de consumo de drogas (Pascual et al., 2009; Montesinos et al., 2016) y produce consecuencias negativas a corto y largo plazo, como el deterioro de la memoria y la muerte de células neuronales en varias regiones del cerebro (Pascual et al., 2007), que son en gran medida irreversibles (Guerri, 2010). La exposición a drogas de abuso puede inducir efectos neuroconductuales, neuroquímicos y neuroendocrinos en el cerebro de ratas adolescentes, afectando así al proceso de crecimiento y a los sistemas implicados en la plasticidad y la cognición (Jain & Balhara, 2010).

Como ya se ha comentado, el estrés es uno de los principales factores de riesgo en el desarrollo de trastornos mentales. Sin embargo, existen algunos rasgos individuales o condiciones internas que pueden activarse ante situaciones amenazantes o estresantes que condicionan el tipo de afrontamiento que se expresa en respuesta al estresor (Dantzer et al., 2018). En consecuencia, algunos individuos son más vulnerables que otros a desarrollar enfermedades relacionadas con el estrés, ya que esto depende, en parte, de las estrategias cognitivas utilizadas para resolver los problemas y de la capacidad para desarrollar de estrategias de resistencia o protección que están vinculadas a variables individuales. La resiliencia se define como la capacidad de los individuos para mantener un funcionamiento psicológico y físico adaptativo y evitar la aparición de enfermedades mentales cuando se exponen a altos niveles de estrés (Charney, 2004). El estudio de la resiliencia es esencialmente fenomenológico, pero ya se están empezando a identificar las características psicológicas y biológicas de los individuos que son más resistentes a las psicopatologías, como la depresión o el TEPT, tras la exposición al estrés (Pfau & Russo, 2015). La mayoría de las investigaciones se han centrado en el estudio de

la resiliencia a los efectos de la evitación social, la anhedonia o las conductas de tipo depresivo (Krishnan et al., 2007). Se han observado numerosos cambios en diferentes sistemas fisiológicos en los sujetos resilientes, como una respuesta menos reactiva del eje HHA, una mayor activación neuronal o un aumento de la señalización glutamatérgica y de la conectividad sináptica. También se han observado diferencias en la transmisión dopaminérgica, caracterizadas principalmente por una excitabilidad intrínseca de las neuronas estimuladas por canales de K+ (Cao et al., 2010; Covington et al., 2010; Christoffel et al., 2012; Linden et al., 2006; Krishnan et al., 2007). No obstante, muchos de los mecanismos implicados en el fenotipo resiliente siguen siendo desconocidos y requieren de mayor estudio. Este desconocimiento es aún mayor en el estudio de la respuesta resiliente al estrés en animales adolescentes. Sólo unos pocos estudios han observado que no existe una respuesta homogénea a las conductas de tipo depresivo, la evitación social y la anhedonia en los adolescentes expuestos al estrés social. Es decir, a diferencia de lo que se observa en roedores adultos, sólo un pequeño porcentaje de los animales era totalmente susceptible o resiliente a estos comportamientos. Esto pone de manifiesto la complejidad de esta etapa del ciclo vital y la dificultad para interpretar los comportamientos observados (Alves-dos-Santos et al., 2020; Vassilev et al., 2021).

Existen múltiples mecanismos implicados en los efectos del estrés social, pero el proceso de neuroinflamación ha cobrado gran relevancia en las últimas décadas, ya que se ha relacionado con el desarrollo de enfermedades mentales y neurodegenerativas. En los años 90 se esbozó la llamada teoría neuroinflamatoria de la depresión (véase, por ejemplo, Maes et al., 2009), que se basa en el aumento de los mediadores inflamatorios observado en pacientes con depresión y en la aparición de depresión o ansiedad en sujetos a los que se les han inyectado citoquinas. El interés por el papel del sistema inmunitario como mediador de los efectos negativos del estrés social ha ido en aumento, y en la actualidad hay numerosos estudios que

demuestran su papel como mecanismo por el que se produce una mayor vulnerabilidad a enfermedades mentales como los trastornos del estado de ánimo y las adicciones (Cathomas et al., 2019). El estrés social influye en la respuesta inmunitaria del organismo, estimulando los leucocitos y la microglía, aumentando las células inflamatorias periféricas y los niveles cerebrales de citocinas, quimiocinas y otros componentes de la respuesta inflamatoria (Calcia et al., 2016; Rodríguez-Arias et al., 2018). Estos marcadores neuroinflamatorios pueden alterar la permeabilidad de la barrera hematoencefálica, permitiendo que las células inflamatorias periféricas penetren en el sistema nervioso central, aumentando así la respuesta inflamatoria (Rodríguez-Arias et al., 2017). La liberación de citocinas y quimiocinas proinflamatorias conduce a su vez a la activación microglial y a la astrogliosis, provocando cambios estructurales y funcionales en el cerebro (Calcia et al., 2016). Dada la importancia de la respuesta inmunitaria en el comportamiento normal y la función neuronal, un desajuste en el funcionamiento del sistema inmunitario inducido por un estrés social prolongado en el tiempo es clave en el desarrollo de trastornos patológicos como la depresión, la ansiedad o el TUS (Reddaway & Brydges, 2020). Se han estudiado principalmente los cambios inducidos por el estrés social a corto plazo en la respuesta inmunitaria, pero algunos estudios muestran que estos cambios pueden ser duraderos y conducir a cambios permanentes en el sistema inmunitario en desarrollo (Lo Iacono and Carola, 2018). Por lo tanto, la respuesta inmune es un mecanismo potencial por el que se puede explicar la mayor vulnerabilidad a la TUS. La respuesta inmunitaria en el sistema nervioso central afecta a la señalización de la DA en la vía mesolímbica, modificando las conductas de recompensa (Thomas Broome et al., 2020). Otro componente de la respuesta neuroinflamatoria estudiado en relación con el abuso de sustancias y la exposición a la derrota social son los receptores TLR4 del sistema inmunitario innato, que reconocen patrones moleculares asociados a patógenos. La exposición a la derrota social activa estos receptores que modulan factores de

transcripción vinculados a la plasticidad neuronal, la memoria y la neurotoxicidad (Jacobsen et al., 2014; Nie et al., 2018). Por último, hay que recordar el potencial neuroinflamatorio del alcohol. El alcohol es capaz de activar la respuesta inflamatoria principalmente a través del reclutamiento de TLR4 de las células gliales, astroglía y microglía para inducir la endocitosis causando la internalización y el tráfico del receptor. La activación de estos receptores por el alcohol promueve la generación de citoquinas proinflamatorias, quimiocinas y mediadores inflamatorios (Pascual et al., 2021). Así, los resultados obtenidos hasta la fecha apuntan al sistema inmune como una importante diana terapéutica para tratar los problemas relacionados con la adicción derivados de la exposición al estrés social.

Cabe destacar que, al contrario de lo que ocurre en los animales susceptibles de presentar conductas de tipo depresivo, los ratones resilientes al estrés social muestran una respuesta neuroinflamatoria menos pronunciada tras la exposición al estrés agudo o crónico, y la aplicación de tratamientos farmacológicos e intervenciones ambientales en roedores susceptibles parece eficaz para reducir esta respuesta (Ballestín et al., 2021; Giménez-Gómez et al. 2021; Hodes et al., 2014; Stewart et al., 2015). Varios estudios preclínicos demuestran que los tratamientos con anti-inflamatorios no esteroideos, como la indometacina, u hormonas como la oxitocina, fueron capaces de amortiguar y prevenir la aparición de los efectos neuroinflamatorios y conductuales de la derrota social (Ferrer-Pérez et al., 2018a, 2021; Giménez-Gómez et al., 2021). En el caso de las intervenciones ambientales, se han evaluado numerosas intervenciones y existe un amplio consenso en la literatura científica de que el ejercicio físico moderado y el enriquecimiento ambiental en el alojamiento de los animales pueden ser estrategias eficaces para reducir la respuesta neuroinflamatoria, las conductas de tipo depresivo y las conductas relacionadas con la adicción inducidas por el estrés social (Hüttenrauch et al, 2016; Kentner et al., 2018; Lynch et al., 2019; Mul et al., 2018; Novkovic et al., 2015; Neal et al., 2018; Pietrelli et al., 2018; Salam et al., 2009; Schloesser et al.,

2010; Zhang et al., 2018; Zolfaghari et al., 2021). Por otro lado, aunque el estrés en edades tempranas (infancia y adolescencia) puede tener consecuencias perjudiciales a largo plazo en la edad adulta, algunos estudios muestran que sí este estrés es moderado puede inducir un fenotipo más resiliente a futuras exposiciones a eventos estresantes. Esta teoría se fomenta en el fenómeno de la inoculación del estrés y, aunque hay pocos estudios hasta la fecha, es una intervención muy prometedora para promover la resiliencia (Dienstbier, 1989; Masten, 2001; Mortimer & Staff, 2004; Rutter, 2006).

OBJETIVOS E HIPÓTESIS

Como hemos comentado, el estrés social es el principal factor de riesgo de las conductas adictivas. En la última década, los fenotipos susceptibles al estrés se han convertido en un objetivo de estudio para la aplicación de tratamientos eficaces para mejorar la resiliencia al estrés. Promover el afrontamiento activo mediante intervenciones farmacológicas y ambientales positivas está validado científicamente en el tratamiento de las adicciones. Sin embargo, estas intervenciones durante la adolescencia para promover la resiliencia como medida preventiva ante experiencias estresantes intensas durante la edad adulta han sido poco estudiadas.

El objetivo principal de la presente Tesis Doctoral fue ***potenciar la resiliencia a los efectos negativos inducidos por la derrota social sobre la conducta adictiva y la respuesta neuroinflamatoria mediante intervenciones preventivas***.

Para evaluar este objetivo, primero confirmamos y caracterizamos el potencial neuroinflamatorio del estrés por derrota social y su mediación en el aumento del consumo de etanol utilizando el paradigma operante de autoadministración oral. Además, quisimos validar el potencial de algunos de los tratamientos más utilizados en la literatura científica, como la administración de oxitocina y el ejercicio físico, para disminuir los efectos negativos sobre el consumo de etanol y la respuesta

inflamatoria inducida por la derrota social. Posteriormente, nos propusimos caracterizar la vulnerabilidad al estrés detectando los ratones susceptibles a la derrota social mediante el Test de Interacción Social (SIT). Una vez caracterizados los fenotipos resilientes/susceptibles a las conductas depresivas inducidas por la derrota social, evaluamos el potencial preventivo de algunas intervenciones ambientales que cuentan con importante evidencia científica, como el ejercicio físico, el enriquecimiento ambiental o la inoculación de estrés durante la adolescencia. A continuación, decidimos caracterizar la vulnerabilidad y resiliencia al estrés en ratones adolescentes, ya que la respuesta a la derrota social es compleja y única durante esta etapa de la vida. Además, se inició una nueva línea de investigación para caracterizar aún más los fenotipos resilientes/susceptibles a las conductas de tipo depresivo, centrándose en el estudio de los cambios morfológicos neuronales y sinápticos y la astrogliosis en estructuras cerebrales clave tanto en la neurobiología del estrés social como en la conducta adictiva.

La identificación de los sujetos más vulnerables a las conductas de tipo depresivo, así como la determinación del potencial de las intervenciones realizadas principalmente durante la adolescencia y antes de las experiencias vitales estresantes en la edad adulta, contribuirán al desarrollo de terapias preventivas y terapéuticas individualizadas en el tratamiento de los trastornos adictivos.

A continuación, se describen los principales objetivos e hipótesis de los siete estudios que componen esta tesis doctoral.

El objetivo del primer estudio fue evaluar si el tratamiento previo a la derrota social con el neuropéptido oxitocina podía proteger contra el consumo de etanol y la respuesta neuroinflamatoria inducida por la derrota social. Para ello, administramos 1 mg/kg de oxitocina media hora antes de cada derrota social. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones derrotados mostrarán un aumento

de los niveles de proteína CX3CL1 y CXCL12 en el estriado después de la cuarta derrota social; 2) La derrota social inducirá un aumento a largo plazo del consumo y la motivación para obtener etanol en la autoadministración oral operante de etanol (6%); 3) Los ratones derrotados mostrarán un aumento de los niveles de proteína CX3CL1 y CXCL12 en el estriado tras la finalización de la autoadministración oral de etanol; 4) La oxitocina evitará el aumento de los niveles de proteína CX3CL1 y CXCL12 en el estriado después de la primera y cuarta derrota social; 5) La oxitocina evitará el aumento del consumo de etanol y de la motivación para obtener la sustancia inducido por la derrota social; 6) La oxitocina evitará el aumento de los niveles de proteína CX3CL1 y CXCL12 en el estriado tras la autoadministración de etanol por vía oral.

El objetivo del segundo estudio fue evaluar si la exposición a la actividad física voluntaria y controlada es una intervención eficaz para prevenir y reducir las consecuencias a largo plazo inducidas por la derrota social en el consumo de etanol y en la respuesta neuroinflamatoria de las quimiocinas CX3CL1 y CXCL12. Para ello, la mitad de la muestra fue sometida a ejercicio físico accediendo a una cinta de correr de bajo perfil 1h al día 3 veces por semana durante todo el procedimiento experimental. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones derrotados mostrarán un aumento de los niveles de proteína CX3CL1 y CXCL12 en el estriado después de la cuarta derrota social; 2) La derrota social inducirá un aumento a largo plazo del consumo y la motivación para obtener etanol en la autoadministración oral operante de etanol (6%); 3) Los ratones derrotados mostrarán un aumento de los niveles de proteína CX3CL1 y CXCL12 en el estriado tras la finalización de la autoadministración oral de etanol; 4) El afrontamiento pasivo durante las sesiones de derrota social se correlacionará con un mayor consumo de etanol durante el programa FR1 de autoadministración oral de etanol; 5) El acceso a la rueda de correr voluntaria contrarrestará el aumento del consumo de etanol y la motivación para obtener la sustancia inducida por la derrota social; 6)

El acceso a las ruedas de correr voluntarias evitará el aumento de los niveles de quimioquinas en el estriado inducido por la derrota social.

El objetivo del tercer estudio fue evaluar el efecto del enriquecimiento ambiental durante la adolescencia sobre las consecuencias conductuales y neuroinflamatorias de la derrota social. Para ello, primero evaluamos y caracterizamos los fenotipos susceptible/resiliente a los efectos conductuales y neuroinflamatorios inducidos por la derrota social. Para clasificar los fenotipos, se evaluó el retraimiento social en la prueba de interacción social 24 h después de la última derrota social. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones sometidos a la derrota social mostrarán un fenotipo resiliente o susceptible en función de sus conductas de evitación o interacción social en el SIT; 2) Los ratones susceptibles a comportamientos de tipo depresivo mostrarán una mayor respuesta en la autoadministración operante de etanol, 3) La derrota social producirá una mayor respuesta neuroinflamatoria en los ratones susceptibles; 4) El enriquecimiento ambiental contrarrestará los efectos inducidos por el estrés social en ratones susceptibles; 5) El enriquecimiento ambiental contrarrestará el aumento de la ingesta de etanol en la autoadministración oral en ratones susceptibles; 6) El enriquecimiento ambiental disminuirá la respuesta neuroinflamatoria de los ratones susceptibles.

El objetivo del cuarto estudio fue evaluar el efecto preventivo del ejercicio físico para promover la resiliencia al aumento del consumo de etanol y los cambios del factor neurotrófico derivado del cerebro (BDNF) inducidos por la derrota social. Para ello, los ratones fueron sometidos a ejercicio físico 1h al día tres veces por semana desde la adolescencia hasta la primera derrota social en la edad adulta. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones susceptibles a comportamientos de tipo depresivo sin acceso al ejercicio físico mostrarán un mayor consumo de etanol y una disminución de los niveles de proteína BDNF en el estriado y el hipocampo; 2) El acceso al ejercicio físico promoverá un aumento en el

porcentaje de ratones resilientes a los comportamientos de tipo depresivo inducidos por la derrota social; 3) El acceso al ejercicio físico disminuirá el consumo de etanol en el paradigma de beber en la oscuridad en ratones susceptibles; 4) El acceso al ejercicio físico disminuirá el consumo de etanol y la motivación por el etanol en la autoadministración oral en ratones susceptibles; 5) El acceso al ejercicio físico restaurará los niveles de proteína BDNF en el estriado y el hipocampo.

El objetivo del quinto estudio era evaluar si la inoculación de un estrés social leve durante la adolescencia puede aumentar la resistencia a otros factores de estrés de mayor intensidad en la edad adulta. Para ello, los ratones adolescentes fueron expuestos a una única derrota social en la adolescencia. Posteriormente, en la edad adulta, los ratones fueron sometidos al protocolo de derrota social. Tres semanas después, se evaluó la autoadministración oral de etanol y se analizó la respuesta inflamatoria de IL-6 y CX3CL1. Para ello, quisimos contrastar las siguientes hipótesis: 1) La inoculación de estrés en la adolescencia aumentará el porcentaje de ratones resilientes a comportamientos de tipo depresivo tras la exposición a la derrota social en la edad adulta; 2) Los ratones susceptibles a comportamientos de tipo depresivo expuestos a la inoculación de estrés reducirán el consumo de etanol en el paradigma de beber en la oscuridad; 3) Los ratones susceptibles a comportamientos de tipo depresivo sometidos a la inoculación de estrés reducirán el consumo de etanol y la motivación en la autoadministración oral inducida por la derrota social; 4) Los ratones susceptibles a comportamientos de tipo depresivo sometidos a la inoculación de estrés mostrarán una menor respuesta neuroinflamatoria inducida por la derrota social.

El objetivo del sexto estudio fue caracterizar el fenotipo resiliente/susceptible en ratones expuestos a la derrota social durante la adolescencia. Se utilizó un procedimiento de derrota social similar al empleado en los estudios anteriores, pero los encuentros con los ratones intrusos tuvieron lugar desde el día postnatal 27 al 36.

Tres semanas después, un conjunto de ratones fue sometido al condicionamiento de preferencia de lugar con 1,5 mg/kg de cocaína y otro conjunto a la autoadministración oral de etanol (6%). Al final de ambos paradigmas, se extrajeron los cerebros y se analizaron los niveles de IL-6 y CX3CL1 en la corteza prefrontal y el estriado para evaluar la respuesta neuroinflamatoria inducida por la derrota social. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones adolescentes mostrarán un fenotipo resiliente o susceptible a los comportamientos de tipo depresivo inducidos por la derrota social en la prueba de interacción social; 2) Los ratones adolescentes susceptibles mostrarán una respuesta ansiógena elevada en comparación con los ratones resilientes; 3) Los ratones adolescentes susceptibles a comportamientos de tipo depresivo mostrarán una mayor respuesta a la recompensa de la cocaína; 4) Los ratones adolescentes susceptibles al comportamiento de tipo depresivo mostrarán un mayor consumo de etanol; 5) En los ratones adolescentes susceptibles se observará una mayor respuesta neuroinflamatoria.

El objetivo del séptimo estudio fue analizar las características morfológicas neuronales y sinápticas y la respuesta astroglial de los individuos susceptibles/resilientes a los efectos negativos de la derrota social sobre las acciones reforzantes del etanol. Por lo tanto, el objetivo era explorar si existen diferencias morfológicas entre los ratones susceptibles y resilientes. Para llevar a cabo este estudio, se enviaron al Instituto de Biología Celular y Neurociencias (IBCN, Buenos Aires, Argentina) cerebros de ratones machos sometidos a la derrota social, clasificados como resilientes/susceptibles según sus puntuaciones en el SIT, y evaluados en el paradigma de autoadministración oral de etanol (20%). Una vez allí, se analizó tanto la morfología sináptica y neuronal como la respuesta astroglial de los animales mediante inmunofluorescencia de varios marcadores (MAP-2, NF200, GFAP y S-100β) en la corteza prelímbica, el estriado y la CA1 del hipocampo. Para ello, quisimos contrastar las siguientes hipótesis: 1) Los ratones susceptibles a comportamientos depresivos inducidos por la derrota

social consumirán mayores cantidades de etanol; 2) Los ratones susceptibles a comportamientos depresivos inducidos por la derrota social mostrarán una disminución de la arborización dendrítica, mostrando una disminución de la inmunotinción MAP-2; 3) Los ratones susceptibles a comportamientos depresivos inducidos por la derrota social mostrarán un citoesqueleto axonal alterado, mostrando una inmunotinción reducida de neurofilamentos NF200; 4) Los ratones susceptibles a comportamientos depresivos inducidos por la derrota social mostrarán un aumento de la astrogliosis, mostrando una mayor inmunotinción de las proteínas GFAP y S-100β.

MATERIAL Y MÉTODOS

En esta tesis doctoral se utilizaron ratones de la cepa C57BL/6J y OF1 (Charles River, Francia). El número total de ratones se especifica en cada uno de los estudios presentados en la sección de Resultados. Los ratones experimentales fueron entregados en nuestro laboratorio en el día postnatal (DPN) 21 (en los estudios 2, 3, 4, 5, 6 y 7) y en el DPN 42 (estudio 1). Todos los ratones (excepto los utilizados como oponentes agresivos) se alojaron en grupos de cuatro o cinco en jaulas de plástico (25 × 25 × 14,5 cm), excepto en el Estudio 3, en el que durante el DPN 21 a 47 se alojaron en jaulas de enriquecimiento ambiental (27 × 27 × 14 cm). En los estudios 1, 2 y 7, se utilizaron ratones de la cepa OF1 como sujetos experimentales y en los estudios 3, 4, 5 y 6, ratones de la cepa C57BL/6J. Los ratones OF1 utilizados como oponentes agresivos fueron alojados individualmente en jaulas de plástico (23 × 13,5 × 13 cm) durante un mes antes de los experimentos para inducir una mayor agresividad (Rodríguez-Arias et al., 1998). Todos los ratones fueron alojados bajo las siguientes condiciones: temperatura y humedad constantes, un ciclo de luz invertido (luces apagadas de 08:00 a 20:00), y comida y agua disponibles *ad libitum*, excepto durante las pruebas de comportamiento. Todos los procedimientos se llevaron a cabo de acuerdo con las directrices de la Directiva del

Consejo Europeo 2010/63/UE que regula la investigación con animales y fueron aprobados por el comité ético local (Universidad de Valencia).

Como metodología principal para inducir estrés social hemos utilizado la derrota social repetida, paradigma ampliamente utilizado en roedores y considerado el más representativo en el estudio de los comportamientos defensivos, de la subordinación y la agresividad. Este modelo de derrota social repetida se compone de 4 sesiones de 25 minutos a intervalos de 72 horas. La fase inicial comienza con la introducción del "intruso" (el animal experimental) en la jaula del "residente" (el oponente agresivo) durante 10 minutos (Tornatzky & Miczek, 1993). Durante esta fase inicial, el intruso está protegido de los ataques mediante una malla metálica, la cual permite las interacciones sociales y las amenazas típicas de la especie por parte del residente agresivo macho, facilitando así la instigación y la provocación (Covington & Miczek, 2001). En la segunda fase, se retira la malla metálica de la jaula para permitir la confrontación entre los dos animales durante un periodo de 5 minutos. Por último, se vuelve a colocar la malla metálica en la jaula para separar a los dos animales una vez más durante otros 10 minutos para permitir las amenazas sociales del residente. Los grupos de exploración sin estrés se someten al mismo protocolo, pero sin la presencia del ratón "residente" en la jaula. El criterio utilizado para definir a un animal como derrotado fue la adopción de una postura específica caracterizada por una posición sumisa erguida, patas delanteras flácidas, cabeza inclinada hacia arriba y orejas retraídas (Miczek et al., 1982; Rodríguez-Arias et al., 1998).

En el estudio 6, se realizó la prueba del laberinto elevado en cruz que consiste en dos brazos abiertos (30 × 5 × 0,25 cm), dos brazos cerrados (30 × 5 × 15 cm) y una plataforma central (5 × 5 cm) elevada 45 cm sobre el nivel del suelo. Para disminuir el estrés experimental, los ratones se habituaron a la sala de experimentación durante 1 hora antes de la prueba. Al principio de cada ensayo, los ratones experimentales se colocaron en la plataforma central frente a un brazo abierto y se les permitió explorar

durante 5 minutos. El comportamiento mostrado por los ratones durante la prueba se registró mediante un sistema de seguimiento automatizado (EthoVision XT 11, Noldus) que registra el número de entradas y el tiempo empleado en cada sección del laberinto (brazos abiertos, brazos cerrados, plataforma central). Se midió el tiempo y el porcentaje de tiempo empleado en los brazos abiertos para caracterizar los efectos ansiolíticos de la derrota social (Ferrer-Pérez et al., 2018b; Rodríguez-Arias et al., 2016).

Para evaluar los fenotipos asociados a las conductas de tipo depresivas inducidas por el estrés social se utilizó el SIT, empleado ampliamente para la detección de la evitación social en roedores estresados. La relación de evitación social utilizada, para definir los fenotipos, se basó en la prueba de aproximación-evitación social descrita previamente por Berton et al. (2006). La prueba se realiza 24 h después de la última derrota durante el ciclo de oscuridad y en un entorno diferente de las sesiones de confrontación. En primer lugar, se realiza un periodo de habituación de 1 h. Después de la habituación, se coloca a cada animal en el centro de una arena cuadrada (campo abierto de plexiglás blanco, de 30 cm de lado y 35 cm de altura) y se monitoriza su comportamiento mediante vídeo (EthoVision XT 11, Noldus). El test consta de dos sesiones con una duración de 600 s cada una. En la primera (sesión de objeto), se coloca una jaula de plexiglás perforada vacía (10×6,5×35 cm) en el centro de una de las paredes de la arena. En la segunda sesión (sesión social), se introduce en la jaula un ratón macho desconocido como estímulo social.

Entre las intervenciones para potenciar la resiliencia se han utilizado, por un lado, intervenciones farmacológicas mediante la administración de oxitocina antes de cada derrota social y, por otro lado, estrategias basadas en la intervención ambiental se utilizaron el ejercicio físico voluntario, el enriquecimiento ambiental y la inoculación de estrés.

En el estudio 1, se aplicó una intervención farmacológica administrando el neuropéptido oxitocina para evaluar su efecto sobre la respuesta neuroinflamatoria inducida por la derrota social tras la primera y la cuarta derrota, y tras el final del paradigma de autoadministración oral de etanol. Para ello, los ratones recibieron una inyección de solución salina o 1 mg/kg de oxitocina 30 min antes de cada derrota social o sesión de exploración.

En los estudios 2 y 4, se utilizó la rueda para correr de bajo perfil (Med Associates Inc.), que gira sobre un eje central en un plano horizontal, lo que permite realizar la actividad física mediante un ejercicio natural como en la locomoción espontánea. Los ratones en la condición de ejercicio fueron colocados individualmente en una jaula de plástico diferente a la de su hogar con una rueda de correr de bajo perfil durante 1h tres veces a la semana (lunes, miércoles y viernes). En el Estudio 2, los ratones en la condición de ejercicio físico tuvieron acceso a la rueda de correr durante todo el procedimiento experimental y las semanas de derrota social tuvieron acceso inmediatamente antes de cada sesión. En cambio, en el Estudio 4, los ratones en la condición de ejercicio físico tuvieron acceso a la rueda de correr sólo entre los DPN 26 y 46, antes del inicio de las sesiones de derrota social.

En el Estudio 3, los ratones en la condición de alojamiento normal se alojaron en grupos de cuatro en jaulas de plástico transparente (27 × 27 × 14 cm) sin más enriquecimiento que el lecho estándar (escamas de madera de 1-3,35 mm), material de anidación (hebras de papel) y dos palos de madera para roer (5 x 1 × 1 cm) por jaula. Los ratones en condiciones de enriquecimiento ambiental se alojaron en grupos de cuatro en jaulas de plástico (59 x 38 × 20 cm) con lecho y material de anidación estándar, dos palos de madera para roer, un túnel adicional de PVC (13 × 5,5 cm) y una casa de plástico para ratones (12,5 x 10,5 × 11 cm; Giménez-Gómez et al., 2021).

En el estudio 5, la mitad de los grupos experimentales fueron sometidos a una única sesión de derrota social el DPN 28. Este encuentro agonístico se llevó a cabo siguiendo el mismo protocolo de derrota social. La única diferencia es que sólo se realizó un encuentro durante la etapa de la adolescencia.

En los estudios 5, 6 y 7, el paradigma del *Drinking in the Dark* se implementó como fase de preexposición al etanol antes de iniciar el procedimiento de autoadministración oral de etanol. Siguiendo el paradigma básico de Rhodes et al. (2005), la prueba consta de dos fases. La primera es la de habituación, en la que se aloja a los animales individualmente 2 h por día para que se habitúen a las jaulas y a las pipetas que se utilizarán durante toda la prueba. En esta fase las pipetas contienen agua y se realiza durante una semana. En la segunda fase del protocolo, la prueba comienza 3 horas después del inicio de la fase oscura del ciclo, y el agua de las pipetas es sustituido por una solución de etanol al 20% (v/v). Tras un periodo de 2 h, los animales son devueltos a sus jaulas agrupadas, con comida y agua *ad libitum* nuevamente. Este procedimiento se repite los días 2 y 3, y el día 4, el procedimiento dura 4 h. Además, inmediatamente después de cada día, se registra el consumo de líquidos. Cada día se prepara una nueva solución de etanol.

Para evaluar el consumo y la motivación por el alcohol se utilizó el paradigma operante de autoadministración oral de etanol. Se llevó a cabo en 8 cámaras operantes modulares (MED Associated Inc., Georgia, VT, USA). Un paquete de software (Cibertec, SA, España) controló la administración de estímulos y fluidos, y registró las respuestas operantes. Las cámaras se colocaron dentro de cajas de aislamiento acústico equipadas con una luz ambiental, dos orificios para introducir el hocico (*nose-pokes*), un receptáculo para dejar caer una solución líquida, una bomba de jeringa, una luz de estímulo y un zumbador. En los estudios 1, 2, 3 y 4, los *nose-pokes* activos suministraron 36 µl de líquido en combinación con una luz de estímulo de 0,5s y un pitido de zumbador de 0,5s, a lo que siguió un periodo de

tiempo de espera de 6s. Los *nose-pokes* inactivos no produjeron ninguna consecuencia.

Para evaluar las consecuencias de la derrota en la adquisición de la autoadministración oral, los animales se sometieron a tres fases experimentales: entrenamiento, sustitución por sacarina y consumo de etanol al 6%. Fase de entrenamiento (8 días): Dos días antes del inicio del experimento, se restringió el acceso a la dieta estándar a 1h al día. Antes de la primera sesión de entrenamiento, se retiró el agua durante 24 horas, y se proporcionó comida 1h antes de la sesión para aumentar la motivación de las respuestas activas. Durante los tres días siguientes, se proporcionó agua *ad libitum*, excepto durante el periodo de 1h de acceso a la comida antes de comenzar cada sesión, en el que se retiró la botella de agua de las jaulas (postprandial). Durante los cuatro días siguientes, y durante el resto del experimento, se proporcionó acceso a la comida durante 1h después del final de cada sesión diaria y el agua estuvo disponible *ad libitum* para evitar el consumo de etanol debido a la sed (preprandial). El programa de restricción de alimentos produjo una pérdida de peso en los ratones de alrededor del 15% de su peso de alimentación libre (Navarrete et al., 2012). Los ratones fueron entrenados para responder al *nose-poke* activo para recibir 36 μl de refuerzo de sacarina al 0,2% (p/v). Sustitución de la sacarina (9 días): La concentración de sacarina se redujo gradualmente a medida que se aumentaba la concentración de etanol (Roberts et al., 2001; Samson, 1986). Cada combinación de solución se estableció en tres sesiones consecutivas por combinación (0,15% Sac - 2% de etanol; 0,10% Sac -4% de etanol; 0,05% Sac -6% de etanol). Consumo de etanol al 6% (11 días): El objetivo de la última fase fue evaluar el número de respuestas activas, la ingesta de etanol al 6% (v/v) y la motivación para beber. Esta fase comenzó 38 días después de la última derrota social. Después de cada sesión, se recogía el alcohol que quedaba en el receptáculo y se medía con una micropipeta. Para lograr este objetivo, durante la última fase se midió el número de respuestas activas y el consumo de etanol (μl) bajo FR1 durante 5 sesiones diarias consecutivas,

bajo FR3 (en la que los ratones tienen que responder tres veces en el *nose-poke* activo para conseguir un refuerzo) durante 5 sesiones diarias consecutivas, y finalmente, al día siguiente de FR3, se realizó una sesión de ratio progresivo (PR) para establecer el punto de ruptura de cada animal (el número máximo de *nose-pokes* que cada animal es capaz de realizar para conseguir un refuerzo). El requisito de respuesta para conseguir refuerzos se incrementó de acuerdo con la siguiente serie: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. Para evaluar la motivación hacia el consumo de etanol, se calculó el punto de ruptura para cada animal como el número máximo de respuestas consecutivas que realizaba para conseguir un refuerzo según la escala anterior. Por ejemplo, si un animal activaba el *nose-poke* un total de 108 veces, esto significaba que era capaz de responder un máximo de 40 veces consecutivas para conseguir un refuerzo. Por lo tanto, el valor del punto de ruptura para este animal sería 40. Todas las sesiones duraron 1 h, excepto la sesión de PR, que duró 2 h (Navarrete et al., 2012, 2014).

En los estudios 5, 6 y 7 se realizaron las siguientes modificaciones. Se suprimió la privación de comida y agua. Por lo tanto, se suprimieron las fases de sustitución por sacarina. Los ratones empezaron la fase de entrenamiento directamente expuestos a una solución de etanol al 20% y los *nose-pokes* activos liberaron 20 µl de solución. Esta fase de entrenamiento duró aproximadamente 12 días. Posteriormente, se inició el programa FR1 durante 10 días (se suprimió el programa FR3). Finalizando el procedimiento con el programa PR. El tiempo de duración de cada fase, las señales ambientales y el funcionamiento de las cajas se mantuvieron como en el protocolo original.

La evaluación del perfil neuroinflamatorio de los ratones se realizó mediante la estimación en tejido cerebral de la concentración de la citoquina pro-inflamatoria IL-6, las quimiocinas CX3CL1 y CXCL12, y el BDNF mediante la técnica de ensayo

por inmunoabsorción ligado a enzimas (ELISA). Para obtener muestras de tejido, los ratones fueron sacrificados por dislocación cervical y luego decapitados. Se extrajeron rápidamente los cerebros y se diseccionaron las estructuras cerebrales con una matriz de corte cerebral con intervalos de corte coronal de 1 mm, utilizando las coordenadas del atlas del cerebro de ratón (Heffner et al., 1980; Franklin & Paxinos, 2008), que luego se conservaron en hielo seco hasta su almacenamiento a -80 °C. Antes de la determinación de IL-6, CX3CL1, CXCL12 y BDNF, los cerebros fueron homogeneizados y preparados siguiendo el procedimiento descrito por Alfonso-Loeches et al. (2010). Las concentraciones de IL-6, CX3CL1, CXCL12 y BDNF en los extractos homogeneizados se midieron con kits comerciales de ensayo inmunoenzimático en placas de 96 pocillos. Todos los reactivos y diluciones estándar se prepararon siguiendo las instrucciones del fabricante. Para determinar la absorbencia, se empleó un lector de microplacas iMark (Bio-RAD) controlado por el software Microplate Manager 6.2. La densidad óptica de las placas se leyó a 450 nm y los resultados finales se calcularon utilizando una curva estándar siguiendo las instrucciones del fabricante. Se determinaron las concentraciones totales de proteínas utilizando el kit de ensayo de proteínas BCA de Pierce (ThermoFisher Scientific) para determinar el número de picogramos de IL-6, CXCL12 y BDNF, y de nanogramos de CX3CL1. Los datos se expresan como pg/mg o ng/mg de proteína para las muestras de tejido.

En el Estudio 7, para el estudio de los cambios morfológicos inducidos por derrota social se empleó la técnica de inmunofluorescencia. Se seleccionaron al azar dos secciones cerebrales coronales de 40 μm de grosor de seis ratones por grupo. Los anticuerpos primarios utilizados fueron: ratón anti-MAP-2 (1:1.000, Sigma-Aldrich, Cat# M4403, RRID:AB_477193), ratón anti- NF200 (1:1.000, Sigma-Aldrich, Cat# N0142, RRID:AB_477257), ratón anti- S-100β (1:1.000, Sigma-Aldrich, Cat# S2532, RRID:AB_477499), conejo anti-GFAP (1:3.000, Agilent Cat# Z0334, RRID:AB_10013382). Los anticuerpos secundarios fluorescentes utilizados fueron:

IgG antiratón conjugado con Alexa FluorTM 568 (1:1.000, Invitrogen, Cat# A11004, RRID:AB_143162) e IgG anti-conejo conjugado con Alexa FluorTM 488 (1:1.000, Invitrogen, Cat# A11008, RRID:AB_143165). Posteriormente, se realizó una contratinción con Hoechst 33342 (1:1.000, Sigma-Aldrich) para marcar los núcleos, se montaron en portaobjetos recubiertos de gelatina y se cubrieron con un medio de montaje de glicerol al 70%. Las fotografías se tomaron en un microscopio invertido Olympus IX83 con un objetivo de 20×. Las imágenes se adquirieron con una cámara digital monocromática sCMOS Orca de alta resolución (Hamamatsu) y el software CellSens Dimension CS-DI-V1. Todas las mediciones se realizaron con el software ImageJ (NIH1). A partir de la inmunotinción, se midió el porcentaje de área cubierta por fibras positivas para MAP-2 y NF200, así como la intensidad óptica de la expresión de S-100β y GFAP en imágenes de 20× de aumento primario.

En relación con la estadística empleada, en los Estudios 3, 4, 5, 6 y 7, los ratones se clasificaron en resilientes y susceptibles en función del SIT. Basándonos en el comportamiento habitual de los ratones C57BL/6 control, los animales con un ratio inferior a 1 se clasifican como susceptibles, mientras que los que tienen un ratio igual o superior a 1 se clasifican como resilientes (Golden et al., 2011). Este criterio fue suficiente para para establecer grupos separados y no se necesitó análisis estadístico adicional. Para analizar las sesiones de derrota social, el procedimiento del *Drinking in the Dark* y la adquisición de la autoadministración oral de etanol, se realizó un ANOVA de medidas repetidas. Además, para analizar el laberinto elevado en cruz, el ratio progresivo, la respuesta neuroinflamatoria, el BDNF y los resultados inmunoquímicos, se realizaron ANOVAs de una vía. En todos los estudios, tras el ANOVA, se calcularon pruebas post-hoc de Bonferroni siempre que fue necesario. Los análisis estadísticos se realizaron con SPSS Statistics (v24 o v.26; IBM, NY, EE.UU.) para los datos conductuales y GraphPad Prism (v8; GraphPad Software Inc., CA, EE.UU.) para el diseño de gráficos. Los datos se

expresaron como media ± SEM y un valor de $p<0,05$ se consideró estadísticamente significativo.

RESULTADOS

En cuanto a los resultados obtenidos, en el primer estudio pudimos corroborar que la derrota social induce un incremento del consumo voluntario de etanol y de la respuesta neuroinflamatoria (evaluando las quimiocinas CX3CL1 y CXCL12) en los ratones adultos sometidos a estrés social. Además, demostramos que la administración del neuropéptido oxitocina antes de cada derrota social evitó el incremento de la respuesta neuroinflamatoria después del primer y cuarto encuentro agonístico. Este efecto preventivo también se observó tras la SA oral de etanol, observándose una disminución de la ingesta de etanol y de la respuesta neuroinflamatoria en los ratones derrotados tratados con oxitocina.

En el segundo estudio observamos como la exposición a ejercicio físico voluntario durante todo el procedimiento experimental resultó efectivo para evitar el desarrollo de los efectos negativos del estrés social sobre el consumo voluntario de etanol y sobre la respuesta neuroinflamatoria. Los ratones tuvieron acceso a las ruedas de correr 1h al día durante 3 días por semana. Fueron sometidos a derrota social al inicio de la adultez y posteriormente, 3 semanas después, fueron expuestos a la AA oral de etanol. Los ratones adultos que tuvieron acceso a la rueda de correr mostraron niveles de consumo de etanol inferiores y una respuesta neuroinflamatoria (niveles de CX3CL1 y CXCL12) reducida en comparación con los ratones sin acceso al ejercicio físico.

En el tercer estudio, nos propusimos caracterizar los fenotipos resiliente/susceptible a las conductas de tipo depresivas inducidas por la derrota social. Mediante el SIT, observamos dos poblaciones y, por tanto, dos respuestas conductuales a los efectos reforzantes del etanol y dos respuestas neuroinflamatorias

diferenciadas. Los ratones susceptibles mostraron un mayor consumo de etanol y una respuesta neuroinflamatoria elevada, mientras que los ratones derrotados resilientes tuvieron un comportamiento y una respuesta neuroinflamatoria similar al grupo control. Adicionalmente y teniendo en cuenta que ciertas intervenciones durante todo el procedimiento experimental son efectivas (Estudio 1) para paliar los efectos conductuales e inflamatorios que induce la derrota social, evaluamos la exposición a un ambiente enriquecido (mediante cajas de alojamiento más grandes con artículos de PVC como casitas y tubos, y material extra de lecho y anidación) solo durante la adolescencia y previo a la exposición de estrés social. Observamos que los animales enriquecidos ambientalmente clasificados como susceptibles a las conductas de tipo depresivas inducidas por la derrota social mostraban un menor consumo de etanol y una respuesta neuroinflamatoria reducida (IL-6 y CX3CL1). Demostrando así que el enriquecimiento ambiental previo a la exposición a derrota social propicia la resiliencia a los efectos conductuales e inflamatorios negativos inducidos por el estrés social.

En el cuarto estudio demostramos el impacto del ejercicio físico durante la adolescencia, previo a la exposición del estrés social sobre los efectos reforzantes y motivacionales del etanol. Los roedores tuvieron acceso a la rueda de correr tres veces por semana durante una hora al día desde el día postnatal 26 hasta el día postnatal 46, es decir durante gran parte de la adolescencia y previo a la exposición a derrota social o exploración. Posteriormente, se aplicó el protocolo de estrés social y se realizó la clasificación de los fenotipos resiliente/susceptibles a las conductas de tipo depresivas inducidas por el estrés social mediante el SIT. El acceso a ejercicio físico no tuvo influencia sobre el porcentaje de ratones resilientes, siendo estos porcentajes similares a los obtenidos en los grupos que no tuvieron acceso a la rueda de correr. A pesar de que el acceso a ejercicio físico se realizó solo durante parte de la adolescencia, se observó que los ratones susceptibles con acceso a la rueda de correr no incrementaron la ingesta ni la motivación por el

etanol. Además, se evaluaron los niveles del BDNF en estriado e hipocampo al final el procedimiento de AA oral de etanol y se observó una disminución de los niveles de proteína en los ratones susceptibles en ambas condiciones (sedentarismo y ejercicio físico voluntario). Por lo que, podemos confirmar que la exposición a ejercicio físico es un potente mecanismo para potenciar la resiliencia y disminuir la vulnerabilidad al incremento de los efectos reforzantes y motivacionales del etanol inducidos por el estrés social. Adicionalmente, corroboramos que el estrés social y la exposición al estrés social producen una disminución de los niveles de BDNF estriatal e hipocampal principalmente en los roedores susceptibles a las conductas de tipo depresivas inducidas por derrota social.

En el quinto estudio, nos propusimos seguir indagando en los diferentes tipos de intervención ambientales. Basándonos en la técnica cognitivo-conductual de entrenamiento por inoculación de estrés (Meichenbaum, 1987, 2017), demostramos que la exposición a un estrés agudo y moderado durante la adolescencia potencia la resiliencia ante otros estresores de mayor intensidad en la adultez. Los ratones susceptibles a las conductas de tipo depresiva mostraron resistencia a los efectos negativos inducidos por la derrota social (respuesta conductual al consumo de etanol y respuesta neuroinflamatoria). Además, la exposición a inoculación de estrés durante la adolescencia incremento el número de ratones con un fenotipo resiliente a las conductas de tipo depresivas en el SIT en comparación con los grupos derrotados no sometidos a inoculación de estrés.

En el sexto estudio nos propusimos caracterizar el fenotipo resiliente a las conductas de tipo depresivas y al aumento en el consumo de etanol en ratones socialmente derrotados durante la adolescencia. Para ello, los ratones adolescentes fueron expuestos derrota social intermitente y, 24 horas después del último encuentro, se les realizó el SIT para evaluar comportamientos de tipo depresivo. Además, se evaluó la conducta ansiógena mediante el Test del Laberinto

Elevado en Cruz. La ingesta de etanol se evaluó 3 semanas después de la última derrota social utilizando la autoadministración oral de etanol. La respuesta neuroinflamatoria se midió al final del procedimiento experimental midiendo los niveles estriatales y corticales de IL-6 y CX3CL1. Los resultados confirmaron que un porcentaje comparable de ratones adolescentes desarrolla resistencia a comportamientos depresivos al observado en ratones adultos. Sin embargo, el aumento de la ansiedad fue más grave en los ratones resilientes. Asimismo, los ratones resilientes bebieron más etanol que los ratones de control. El aumento de IL-6 y CX3CL1 se observó principalmente en el cuerpo estriado de los ratones susceptibles en comparación con el de los ratones de control. Nuestros resultados confirman que, contrariamente a las suposiciones previas en adultos, las respuestas al estrés social son más complejas y singulares en adolescentes, y se debe tener precaución para la correcta interpretación y traducción de esos fenotipos.

En el séptimo y último estudio, nos propusimos dar un paso más allá en el estudio de los efectos de la derrota social sobre la morfología neuronal y sináptica y la astrogliosis. Este estudio se realizó en el Laboratorio de Neurotoxicidad, Neuroprotección y Neurorreparación (Facultad de Medicina, Universidad de Buenos Aires, CONICET, IBCN, Argentina). En este estudio realizamos una evaluación morfométrica de la MAP-2, NF200, GFAP y S-100β en corteza prelímbica, estriado y CA1 del hipocampo. Los ratones fueron sometidos a derrota social durante el inicio de la adultez, se clasificó el fenotipo en relación a las conductas de tipo depresivas y tres semanas después se les expuso a la AA oral de etanol. Al finalizar el procedimiento se procedió al envío de los cerebros al laboratorio de destino para su posterior análisis. Los resultados obtenidos demuestran que la derrota social puede producir cambios en la citoarquitectura neuronal, lo que puede afectar la formación y estabilidad de las conexiones sinápticas en ratones con un fenotipo susceptible. Por el contrario, el aumento del área cubierta por neurofilamentos en la corteza prelímbica de ratones resilientes puede indicar una mayor plasticidad neuronal en

estos individuos. Además, la respuesta de astrogliosis observada en este estudio indica que el estrés social intermitente y la exposición al etanol en los ratones susceptibles pueden producir daño neuronal o una mayor respuesta neuroinflamatoria.

CONCLUSIONES FINALES

Los resultados obtenidos en la presente Tesis Doctoral nos permiten confirmar que la derrota social contribuye al desarrollo de conductas de evitación social e incrementa el consumo de etanol a largo plazo mediante la activación del sistema inmune. Hemos confirmado la existencia de fenotipos diferenciados en base a las respuestas conductuales de evitación social inducidas por la derrota social. El estrés social produce efectos negativos, pero no en todos los animales expuestos, ya que solo un porcentaje de ellos desarrolla un perfil susceptible a las conductas de tipo depresivo e incrementa el consumo de alcohol. En esta Tesis Doctoral hemos caracterizado a los animales susceptible y resilientes a los efectos de la derrota social, tanto cuando esta se produce en la edad adulta como cuando se experimenta en la adolescencia. Pero nuestro principal objetivo ha sido potenciar la respuesta de resiliencia y hemos demostrado que intervenciones farmacológicas como la administración de oxitocina o ambientales como el ejercicio físico pueden potenciar dicha respuesta resiliente. Pero estas intervenciones también pueden aplicarse con carácter preventivo durante la adolescencia. Así por ejemplo el ejercicio físico, el enriquecimiento ambiental o la exposición a un estresor social de baja intensidad antes de experimentar la derrota social inducen una potente respuesta resiliente. Todos estos efectos protectores pueden producirse por diversos mecanismos, algunos de ellos estudiados en esta Tesis Doctoral, como los cambios en el BDNF o en la arquitectura neuronal. Sin embargo, el mecanismo fundamental que aparece como común en los animales resilientes es una disminución de la respuesta

neuroinflamatoria que se observa siempre incrementada en animales derrotados susceptibles.

A continuación, desarrollaremos las principales conclusiones derivadas de la presente Tesis Doctoral.

La derrota social incrementa el consumo y la motivación por el alcohol

Nuestros resultados indican que la derrota social incrementa la vulnerabilidad al desarrollo de conductas adictivas. Al igual que habíamos observado con la cocaína, los ratones derrotados muestran un incremento en el consumo de alcohol y un aumento de la motivación por conseguir una dosis de esta sustancia en el paradigma operante de autoadministración oral de etanol, en comparación con los ratones que fueron expuestos a la condición de exploración y que no experimentaros estrés social. Tanto durante las fases de ratio fijo 1 como 3, los animales derrotados consumieron más alcohol que los controles y realizaron un mayor número de respuestas efectivas. Igualmente, en la prueba de ratio progresiva realizaron un mayor trabajo para conseguir el alcohol que los animales controles. Todos estos efectos los hemos observado 3 semanas después de la última derrota social, por lo tanto, son efectos a largo plazo lo cual incrementa mucho más su valor traslacional puesto que nos indica que el estrés social es capaz de inducir modificaciones que perduran en el cerebro de estos animales a lo largo del tiempo y que van a incrementar la vulnerabilidad a desarrollar una conducta adictiva.

Finalmente, también hemos confirmado que estos efectos se observan igualmente en animales derrotados socialmente durante adolescencia y que se evalúan ya en la edad adulta. Por lo tanto, nuestro modelo ha demostrado ser igualmente válido en animales tanto adultos como adolescentes, lo cual significa que puede considerarse como un buen modelo de bullying o acoso escolar.

Los animales resilientes a las conductas depresivas inducidas por la derrota social también se muestran resilientes al incremento en el consumo de etanol cuando el estrés social ocurre en la edad adulta

Uno de nuestros principales resultados es la confirmación de la existencia de dos fenotipos diferenciados en base a la respuesta de evitación social inducida por la derrota social. El estrés social produce efectos negativos, pero no en todos los animales expuestos, ya que solo un porcentaje de ellos desarrolla retraimiento social (evaluado por el SIT) que se considera una conducta de tipo depresivo. Nuestros resultados han confirmado que aquellos animales susceptibles que desarrollan retraimiento social (depresión) también presentan un incremento en el consumo de etanol. Según los resultados obtenidos en los estudios que componen esta Tesis Doctoral, los ratones susceptibles al estrés social se caracterizaron por desarrollar una respuesta de evitación social mayor tras la derrota social, un aumento en la ingesta de alcohol y una mayor motivación por obtener esta sustancia. Mientras que los ratones resilientes a las conductas de tipo depresivos mostraron una mayor interacción social, menores niveles de consumo y de motivación por el alcohol.

Igualmente, los resultados obtenidos en esta Tesis Doctoral confirman y extienden resultados previos obtenidos en nuestro laboratorio describiendo el diferente afrontamiento que presentan los ratones resilientes y susceptibles durante el encuentro agonístico (Ballestín et al., 2021). Durante el encuentro agonístico, los ratones susceptibles sometidos a cuatro sesiones de derrota social adoptan un perfil de afrontamiento pasivo-reactivo durante los encuentros de derrota social, empleando más tiempo en comportamientos de huida/evitación y sumisión/defensa durante la derrota social. Los ratones derrotados susceptibles mostraron una disminución del tiempo de evitación y huida, y un incremento de las conductas de sumisión y defensa en el cuarto encuentro, lo que indica una falta de flexibilidad

conductual ante la experiencia de derrota social ineludible (Hawley et al., 2010; Lambert et al., 2014). Por el contrario, en algunos grupos de animales hemos observado que los ratones clasificados como resilientes mostraron un menor afrontamiento pasivo-reactivo en comparación con el grupo susceptible, es decir, mostraron mayor afrontamiento activo (Ballestín et al., 2021). Aunque, se requieren más estudios para concluir si las conductas realizadas durante los encuentros agonísticos pueden definir un perfil resiliente o los comportamientos pasivo-reactivos son generales en ambos fenotipos.

Como aportación altamente original, en esta Tesis Doctoral hemos observado que los ratones susceptibles también presentan alteraciones morfológicas en las prolongaciones dendríticas y en el citoesqueleto axonal, que podrían indicar un deterioro de conexiones sinápticas preexistentes y, a su vez, influir en el desarrollo de conductas de tipo depresivo y en la vulnerabilidad al consumo de alcohol. Por el contrario, en los ratones resilientes al estrés se observó un incremento del área cubierta de proteínas presentes en las prolongaciones dendríticas (MAP-2+) y en el citoesqueleto axonal (NF200+) en estriado y corteza prelímbica, respectivamente. Este hallazgo podría indicar una mayor plasticidad neuronal en los individuos resilientes. También, observamos ciertas alteraciones en la respuesta de la gliosis neuronal. La respuesta de astrogliosis observada nos indica que el estrés social repetido y la exposición al alcohol pueden producir daños neuronales o una mayor respuesta neuroinflamatoria en los individuos susceptibles al estrés social.

Los animales resilientes que experimentan la derrota social durante la adolescencia muestran un fenotipo mucho más complejo que los derrotados en la edad adulta

Un interesante resultado de esta Tesis Doctoral ha sido que la experiencia de la derrota social durante la adolescencia presenta características específicas que la

diferencia de la experimentada durante la edad adulta. De acuerdo con los pocos estudios realizados en esta área, observamos que los ratones adolescentes derrotados no desarrollaron un fenotipo general de resiliencia/susceptibilidad. No hay correlación entre la resiliencia a la evitación social y el aumento de la respuesta al alcohol. Aunque el porcentaje de ratones resilientes/susceptibles tras la derrota social es comparable al observado en los ratones adultos (según su respuesta de retraimiento social con el SIT), en los ratones resilientes al desarrollo de la evitación social se observó un aumento de la conducta ansiógena o de la ingesta de alcohol. Solo un 20% del total de los ratones derrotados fue resiliente a los efectos depresivos y el aumento de la ingesta de alcohol inducidos por derrota social. Estos resultados indican que la edad de exposición a la derrota social afecta al desarrollo de la resiliencia y que existe un desarrollo variable de la resiliencia entre los comportamientos de tipo depresivo y la respuesta a la recompensa de la droga. En consonancia con nuestros resultados, Alves-dos-Santos et al. (2020) observaron que los ratones adolescentes derrotados resilientes a la anhedonia o a la evitación social eran los ratones más afectados en términos de resultados endocrinos/fisiológicos (aumento de peso corporal y respuesta a la corticosterona). Asimismo, Vassilev et al. (2021) observaron que, en la adolescencia, la derrota social produce un deterioro del control inhibitorio independientemente de la evitación social.

La resiliencia debe considerarse como un proceso activo, que afecta tanto a las estrategias pasivas como a las activas, para lograr la mayor adaptación al estrés (Russo et al., 2012). Las respuestas en cada sistema particular pueden desarrollarse de forma diferente tras la exposición al estrés (Smith, 2019). Nuestros resultados sugieren que la derrota social durante la adolescencia conduce a un fenotipo propenso a la adicción en algunos ratones, que se manifiestan como resiliente durante el SIT. Aunque es necesario profundizar más en este tema, se han observado cambios en la conectividad dopaminérgica de la corteza prefrontal (Vassilev et al., 2021) que

podrían estar relacionadas con la mayor respuesta a los efectos reforzantes del alcohol observados en los ratones adolescentes resilientes al estrés social.

Nuestros resultados confirman que, al contrario de lo que se suponía en los adultos, las respuestas al estrés de la derrota social son más complejas y singulares en los adolescentes, por lo que hay que tener precaución en la correcta interpretación y traducción de estos fenotipos.

La administración de oxitocina y el ejercicio físico durante la exposición a la derrota social incrementan la respuesta de resiliencia

Una vez confirmada la existencia de dos subpoblaciones entre los individuos sometidos a derrota social y conocida la respuesta a los efectos reforzantes del etanol de cada una de ellas, nos propusimos investigar intervenciones que podrían potenciar la resiliencia para llevar a cabo el objetivo principal de la presente Tesis Doctoral, desarrollar estrategias farmacológicas y ambientales para mejorar la resiliencia a los efectos negativos inducidos por la derrota social sobre el comportamiento y la respuesta neuroinflamatoria.

Para ello, en primer lugar, nos propusimos evaluar intervenciones que actuaran durante las sesiones de derrota social. Por un lado, evaluamos el efecto de la administración de oxitocina antes de cada encuentro agonístico y, por otro, el efecto del ejercicio físico voluntario y controlado durante las sesiones de derrota social pero que en este caso continuara durante el resto del procedimiento experimental. Como primer y crítica condición en estos estudios, comprobamos que las derrotas sociales se produjeron de forma similar en aquellos animales tratados con oxitocina, así como en los expuestos al protocolo de ejercicio físico. Es decir, los animales residentes atacaron y amenazaron con la misma intensidad a los animales intrusos. Igualmente, ninguna de las intervenciones afectó a los comportamientos de evitación/huida y defensa/sumisión d ellos ratones intrusos. Pero lo realmente importante es que sí

observamos diferencias posteriormente en el paradigma de la autoadministración oral de etanol. Los ratones derrotados socialmente, expuestos a estas intervenciones (ya sea la administración de oxitocina antes de cada derrota social o al ejercicio físico) mostraron un menor número de respuestas activas, un menor consumo de alcohol y un menor esfuerzo y motivación por conseguir la droga en comparación con los roedores derrotados sin intervenciones.

El neuropéptido oxitocina actúa como neuromodulador del sistema nervioso central, interviene en una amplia variedad de interacciones sociales, tiene efectos ansiolíticos y atenúa la respuesta del eje HHA al estrés, promoviendo un aumento de la interacción social y disminuyendo la anhedonia y la evitación social que caracteriza al estrés social (Borland et al., 2018; Ebert & Brüne, 2018; Ferrer-Pérez et al., 2019a; Lukas et al., 2011; Nasanbuyan et al., 2018; Steinman et al., 2016; Wang et al., 2018; Winter & Jurek, 2019). Además, se ha observado que la administración de oxitocina disminuye el consumo de drogas, los síntomas de abstinencia y las conductas de búsqueda asociadas a diversas drogas de abuso (Leong et al., 2018; Pedersen, 2017). Por tanto, la administración de oxitocina previa a la exposición a la derrota social podría actuar evitando la sensibilización del sistema de recompensa y así reduciendo los efectos reforzantes del alcohol, por ejemplo, debido a su efecto atenuante sobre la respuesta al estrés.

El ejercicio físico moderado y controlado parece ser un modulador de las funciones mentales superiores. En roedores produce una mejora del aprendizaje, de la neurogénesis, de la angiogénesis, un aumento de los factores neurotróficos induciendo cambios en varias moléculas de señalización, así como reduciendo las conductas asociadas al estrés (Salam et al., 2009; Mul, 2018). Se ha observado que el ejercicio físico tras la derrota social reduce la evitación social y la anhedonia en roedores (Mul et al., 2018; Watanasriyakul et al., 2018; Zhang et al., 2019). Además, el ejercicio físico regula algunos componentes del eje HHA, generando una respuesta

adaptativa al estrés (Pietrelli et al., 2018) y se han observado disminuciones en la ingesta de alcohol cuando se tenía acceso a las ruedas de ejercicio (Ehringer et al., 2009; Darlington et al., 2014, 2016). Se puede concluir que nuestros resultados confirman que el ejercicio físico voluntario y controlado es una herramienta eficaz para prevenir y reducir los efectos perjudiciales relacionados con la conducta adictiva inducidos por el estrés social, fomentando una respuesta neuroendrocrina adaptativa y promoviendo factores neutróficos.

La resiliencia puede potenciarse antes de que se produzca la derrota social

En una serie de estudios posteriores nos propusimos evaluar si determinadas intervenciones ambientales podrían ser útiles como herramientas preventivas y potenciar la respuesta resiliente al estrés. Para evaluar el carácter potenciador y preventivo de estas intervenciones ambientales, todas ellas se realizaron durante el periodo de la adolescencia y finalizaron antes del inicio de la primera exposición a la derrota social, en el día postnatal 47, momento en que se considera a los roedores como jóvenes adultos. Para realizar este objetivo, evaluamos tres condiciones ambientales que tradicionalmente han demostrado ser útiles para controlar los efectos nocivos del estrés: el enriquecimiento ambiental, el ejercicio físico voluntario y la inoculación de estrés.

El enriquecimiento ambiental y el ejercicio físico previo a las derrotas sociales durante la adolescencia no aumentó el porcentaje de ratones resilientes a las conductas de tipo depresivo evaluadas el SIT tras la derrota social. Por el contrario, sí observamos un ligero aumento del porcentaje de ratones resilientes en el grupo de inoculación de estrés. En relación con el consumo de alcohol, las tres intervenciones fueron eficaces para reducir el consumo y la motivación por el alcohol en los animales adultos. En la prueba del Drinking in the Dark observamos una disminución del consumo de alcohol de forma generalizada en los grupos de animales expuestos a ejercicio físico y en los grupos expuestos a inoculación de estrés durante la

adolescencia. En el paradigma operante de autoadministración oral de etanol, se observó que las tres intervenciones eran eficaces para reducir las respuestas activas, el consumo voluntario y el esfuerzo y la motivación para conseguir una dosis de alcohol tanto en los ratones resilientes como susceptibles expuestos a las diferentes intervenciones.

El enriquecimiento ambiental se ha asociado típicamente con una mejora del bienestar, un aumento de la función cognitiva y una potenciación de la resiliencia al estrés. Se han utilizado diferentes modelos para reducir la vulnerabilidad a los efectos perjudiciales de la derrota social, pero los resultados observados en la literatura científica son contradictorios. Algunos estudios han observado aumentos en la agresividad y la ansiedad, con incrementos en los niveles de factor liberador de corticotropina (McQuaid et al., 2013a, 2013b). Sin embargo, otros estudios han mostrado una reducción de la ansiedad, de las alteraciones cognitivas y de los niveles de corticosterona en animales alojados en situación de enriquecimiento ambiental (Bahi, 2017; Branchi et al., 2013; Cordner & Tamashiro, 2016; Dandi et al., 2018; Marianno et al., 2017; Mesa-Gresa et al., 2016; Reichmann et al., 2013). En nuestro laboratorio hemos observado que el tipo de casas y tubos utilizados en el enriquecimiento puede determinar un incremento del estrés en los animales. Debemos tener en cuenta que la edad de los roedores influye en la estabilidad social, ya que a mayor edad y maduración sexual se presentan conductas más agresivas y dominantes en los ratones macho. En cambio, durante el periodo de adolescencia, los ratones, al igual que los humanos, buscan una mayor interacción social y la jerarquía social propia de la adultez aún no se ha consolidado (Bell, 2018; Lo Iacono & Carola, 2018). Por este motivo, consideramos apropiada la utilización de un modelo de enriquecimiento ambiental durante la adolescencia, con el objetivo de moldear un cerebro aún en desarrollo y promover respuestas más resilientes. El enriquecimiento ambiental promueve el desarrollo de respuestas neuroendocrinas

adaptativas al estrés social, lo que indica la importancia de la exposición a entornos complejos durante la adolescencia.

Como se ha discutido en el apartado anterior, el ejercicio físico es capaz de modular factores neurotróficos que inducen cambios positivos y reduce la activación del eje HHA. Teniendo en cuenta estos datos, podemos hipotetizar que estas neuroadaptaciones inducidas en el eje HHA y en los factores neurotróficos se mantienen en el tiempo y median en la repuesta adaptativa al estrés y en el buen funcionamiento del sistema de la recompensa. Gracias a los resultados de esta Tesis Doctoral, podemos confirmar que la pre-exposición voluntaria al ejercicio físico es eficaz para evitar el aumento del consumo y la motivación por el alcohol inducido por la derrota social a largo plazo.

Por otro lado, y a pesar de sus consecuencias negativas, el estrés puede presentar un efecto adaptativo, ya que promueve la homeostasis (Selye, 1975a, 1975b). La exposición a eventos estresantes que no son devastadores, pero que son lo suficientemente desafiantes como para provocar la instigación emocional y el procesamiento cognitivo, puede promover el afrontamiento exitoso de los estresores posteriores (Levine, 1957; Levine et al., 1967; Lyons et al., 2010; Meichenbaum, 2017). Los resultados obtenidos en relación con la exposición a inoculación de estrés durante la adolescencia corroboran la hipótesis de que la inoculación de estrés puede ser un factor protector ante futuras exposiciones a un estresor de mayor intensidad. Basándonos en la poca bibliografía referente a este modelo, hipotetizamos que la inoculación de estrés durante la adolescencia podría aumentar la adaptación al estrés social en cuanto al desarrollo de comportamientos de tipo depresivo en la edad adulta. La inoculación de estrés actuaría como un factor protector, como un mecanismo que prepara al individuo y ayuda en la regulación homeostática del organismo ante futuras exposiciones a estresores de mayor intensidad. Nuestros

resultados han confirmado que este efecto protector se manifiesta sobre todo disminuyendo el consumo de alcohol en la edad adulta.

La resiliencia puede potenciarse antes de que se produzca la derrota social

De manera consistente, en todos los estudios que componen esta Tesis Doctoral, hemos confirmado que la derrota social puede contribuir a potenciar las propiedades gratificantes y motivacionales del alcohol a largo plazo mediante la activación temprana del sistema inmune, provocando un estado de neuroinflamación que contribuye al desarrollo de un perfil vulnerable al trastorno por abuso de sustancias.

Hemos observado como la derrota social repetida induce un incremento de la respuesta neuroinflamatoria. La derrota social incrementa los niveles de las quimiocinas CX3CL1 y CXCL12 en el estriado de los ratones OF1 tras la cuarta derrota social. Además, este efecto sobre la respuesta neuroinflamatoria se mantuvo a largo plazo, ya que los ratones sometidos a derrota social presentaron incrementos de los niveles de ambas quimiocinas en el estriado tras finalizar el paradigma de la autoadministración oral de etanol.

Como ya se ha mencionado, no todos los sujetos experimentales presentaron un fenotipo susceptible al estrés social. Un porcentaje de los ratones es resiliente a las respuestas conductuales, pero, también al incremento en la neuroinflamación inducida por la derrota social. Observamos de forma generalizada incrementos significativos de los niveles de la citocina proinflamatoria IL-6 en estriado y corteza prefrontal solo en los ratones clasificados como susceptibles a las conductas de tipo depresivas inducidas por la derrota social. Por otro lado, en estos estudios hemos observado ciertas discrepancias en la cepa C57BL/6 en relación con la concentración de los niveles de la quimiocina CX3CL1. No existe un consenso claro sobre si la CX3CL1 es una quimiocina anti-inflamatoria o proinflamatoria. La CX3CL1 señaliza a través de su receptor Cx3cr1 que solo se expresa en la microglía, siendo

crítico para la comunicación cruzada microglía-neurona. Los cambios inducidos por el estrés social en CX3CL1 no están claros, algunos estudios reportan disminuciones de la expresión de CX3CL1 en corteza prefrontal, en estriado, hipocampo e hipotálamo (Ballestín et al., 2021; Montagud-Romero et al., 2020; Rosseti et al., 2016; Wholeb et al., 2013) e incrementos de la expresión en el hipocampo dorsal, corteza orbitofrontral y núcleo paraventricular del hipotálamo (Bollinger et al., 2017; Rossetti et al., 2016; Wu et al., 2020). En primer lugar, hemos observado diferencias entre la respuesta a esta quimiocina tras la derrota social dependiendo de la cepa de ratón utilizada. Se han observado incrementos en ratones de la cepa OF1, pero disminuciones en aquellos pertenecientes a la cepa C57BL/6. Para comparar estos estudios, debemos tener en cuenta las diferencias entre estas cepas con respecto a su respuesta al estrés. Los machos OF1 son mucho más territoriales, siendo su repercusión fisiológica mayor tras la derrota. Pero, también observamos además discrepancia en cuanto a la respuesta a los niveles de CX3CL1 en animales C57BL/6 tras la derrota social. Mientras que en el estudio 3 observamos una disminución de los niveles de CX3CL1 en estriado y corteza prefrontal en los ratones susceptibles, por el contrario, en el estudio 5 no observamos diferencias en el estriado, pero sí un incremento de la concentración de CX3CL1 en la corteza prefrontal de los ratones susceptibles. Estos resultados contradictorios confirman la necesidad de continuar estudiando el papel y el funcionamiento de esta interesante quimiocina.

En relación con la respuesta neuroinflamatoria observada en los ratones expuestos a derrota social repetida durante la adolescencia, debemos destacar que, igualmente se observaron aumentos en la concentración de IL-6 y CX3CL1 principalmente en el estriado de los ratones susceptibles. Estos resultados junto a los resultados obtenidos en el estudio 5 en relación con la respuesta neuroinflamatoria del grupo control sometido a inoculación de estrés durante la adolescencia, nos permiten concluir que el estrés social, además de sensibilizar el sistema mesolímbico, sensibiliza el sistema inmunológico durante la adolescencia. Por lo que la derrota social induce un estado

neuroinflamatorio a largo plazo independientemente de la edad de exposición al estrés social.

Entre las numerosas aportaciones de esta Tesis Doctoral, quizá consideramos la más relevante la confirmación de que el sistema inmune podría ser un mecanismo clave mediante el cual los efectos de la derrota social inducen vulnerabilidad en el desarrollo de conductas adictivas. Pero, aún más importante es que mediante la aplicación de diferentes intervenciones hemos podido revertir y evitar la activación de esta respuesta neuroinflamatoria inducida por la derrota social. Numerosos estudios asocian a la oxitocina como un modulador de la respuesta neuroinflamatoria inducida por la derrota social. La oxitocina inhibe mediadores pro-inflamatorios como el TNF-α, la IL-1β, COX-2 y el óxido nítrico sintasa (Akman et al., 2015; Inoue et al., 2019; Karelina et al., 2011; Yuan et al., 2016). En concordancia con lo observado en la literatura científica, mediante la administración previa de oxitocina en cada derrota social, conseguimos bloquear el incremento de los niveles de las proteínas CX3CL1 y CXCL12 en el estriado inducido por la derrota social. Esta disminución de los niveles de concentración se mantuvo en el tiempo, tras el paradigma de la autoadministración oral de etanol. Por tanto, podemos confirmar que la administración exógena de oxitocina tiene efectos antiinflamatorios sobre la respuesta neuroinflamatoria desencadenada por la derrota social.

La literatura científica sugiere que el ejercicio físico de intensidad moderada puede ser óptimo para disminuir los marcadores neuroinflamatorios (Henrique et al., 2018; Paolucci et al., 2018). Algunos estudios han mostrado los efectos positivos del ejercicio físico controlado sobre el estrés (Mul et al., 2018; Ignácio et al., 2019) y la conducta adictiva (Somkuwar et al., 2016). El ejercicio físico interactúa con el estrés y la neuroinflamación en función de la intensidad. Varios estudios han observado que el ejercicio físico controlado reduce los niveles de corticosterona y los receptores de glucocorticoides, atenuando los efectos negativos del estrés crónico

(Zheng et al., 2006; Ignácio et al., 2019; Lynch et al., 2019; Watanasriyakul et al., 2019). La inhibición del exceso de producción de corticosterona puede atenuar la respuesta inflamatoria inducida por el estrés (Niraula et al., 2018). En esta Tesis Doctoral, demostramos que la exposición a ejercicio físico voluntario y controlado durante todo el procedimiento experimental es efectivo para revertir el aumento de los niveles de CX3CL1 y CXCL12 en el estriado inducido por derrota social repetida tras la autoadministración oral de etanol. Por lo tanto, el ejercicio físico voluntario y controlad es una herramienta eficaz para prevenir y revertir la activación del proceso neuroinflamatorio inducido por la derrota social.

Además, observamos un efecto preventivo en la activación de la respuesta neuroinflamatoria en los roedores susceptibles que fueron expuestos a enriquecimiento ambiental. Observamos un efecto generalizado de disminución en la concentración de IL-6 en el estriado en todos los ratones expuestos a enriquecimiento ambiental. Además, en los ratones susceptibles a los efectos de la derrota social expuestos a enriquecimiento ambiental se observó una disminución de los niveles de CX3CL1 en el estriado en comparación con los ratones susceptibles sin enriquecimiento ambiental. Aunque no existen estudios similares, estos resultados van en la línea de un estudio que evaluó el efecto del enriquecimiento ambiental en la respuesta neuroinflamatoria inducida por estrés social moderado, observándose una atenuación del aumento de la expresión del ARNm prefrontal de IL-6 e IL-β1 (McQuaid et al., 2018). Estos resultados sugieren que la exposición a enriquecimiento ambiental antes de la derrota social reduce el impacto del estrés social a largo plazo en la respuesta neuroinflamatoria, actuando como un factor protector.

Por otro lado, evaluando la inoculación de estrés durante la adolescencia, observamos una disminución generalizada de los niveles de IL-6 en la corteza prefrontal de todos los ratones expuestos a inoculación de estrés en comparación a

todos los ratones no inoculados. Adicionalmente, en la corteza prefrontal se observó una disminución de esta citocina entre los ratones susceptibles inoculados con estrés en comparación con los ratones susceptibles no inoculados. Con respecto a la quimiocina CX3CL1, también observamos un papel protector de la inoculación de estrés, ya que observamos una disminución de los niveles en el grupo susceptible expuesto a inoculación de estrés en comparación con el grupo susceptible no expuesto a inoculación en la corteza prefrontal. Además, se observaron diferencias significativas entre los grupos control, con un aumento de los niveles de CX3CL1 en el grupo control expuesto a inoculación de estrés en comparación con el grupo control sin inoculación de estrés. Por el contrario, no se observaron diferencias estadísticas en el estriado. Aunque no existen estudios que apoyen nuestros resultados, consideramos que el modelo de inoculación de estrés es realmente prometedor para prevenir la activación de la respuesta neuroinflamatoria inducida por estrés social, ya que la inoculación de estrés podría inducir una menor reactividad del eje HPA a una experiencia de estrés posterior y/o al desarrollo de una mayor neuroplasticidad o flexibilidad conductual que caracteriza al fenotipo resiliente (Hawley et al., 2010; Lambert et al., 2014; Lee et al., 2014; Macrí et al., 2011). Estos resultados indican que entrenando a los individuos ante la exposición a situaciones estresantes se pueden desarrollar respuestas adaptativas al estrés social.

Limitaciones y estudios futuros

Aunque en esta Tesis Doctoral hemos podido dar respuesta a algunas preguntas muy relevantes, no se encuentra exenta de limitaciones. El fenómeno de la resiliencia es un concepto que aún se encuentra en pleno proceso de investigación, los mecanismos neurobiológicos subyacentes a este fenómeno aún son desconocidos y es necesario realizar más investigaciones y superar algunas limitaciones presentadas en este trabajo. A continuación, se exponen las cuestiones abiertas y los nuevos retos que podrían abordarse en futuros estudios.

Actualmente, sabemos que existen diferencias de género/sexo en la respuesta al estrés y en la vulnerabilidad a trastornos relacionados con el estado de ánimo y a la adicción. Consideramos imprescindible para paliar la principal limitación de este trabajo, y así, ampliar el conocimiento sobre las bases neurobiológicas que subyacen a la resiliencia al estrés y como siguiente paso en la ampliación de esta Tesis Doctoral, la realización de estos estudios en roedores hembra. Actualmente en nuestro laboratorio hemos desarrollado un protocolo de derrota social vicaria en hembras que ha producido efectos muy similares a los observados en machos.

En segundo lugar, proponemos seguir estudiando el potencial preventivo de intervenciones ambientales para potenciar la resiliencia en los roedores adolescentes. En este sentido, hemos demostrado que la caracterización del fenotipo resiliente durante la adolescencia es diferente al presentado en los ratones adultos. La respuesta al estrés por derrota social parece ser compleja y singular en los adolescentes, y se debe ser cuidados en la interpretación y traducción de los fenotipos resilientes/susceptibles. No podemos presuponer que la resiliencia a un fenotipo se desarrolla igualmente para otros cuando se trata de animales adolescentes. En futuros estudios pretendemos desarrollar estrategias para potenciar la resiliencia al estrés social experimentado durante la adolescencia. Algunos de los objetivos preliminares son el estudio de la inoculación de estrés en etapas previas al destete de los ratones mediante la deprivación maternal breve, o la aplicación de ambientes enriquecidos (jaulas más grandes con casitas, tubos de PVC, material extra de anidación, etc.,).

Valor traslacional de este estudio

Los modelos animales son una potente herramienta para realizar investigaciones en diversos ámbitos. En nuestro caso la utilización de modelos animales es fundamental para investigar las bases neurales y fisiológicas del estrés y la adicción a drogas. Sin embargo, cuando nos enfrentamos a unos resultados obtenidos gracias al uso de modelos animales debemos ser prudentes a la hora de trasladarlos a la conducta

humana. La literatura coincide en señalar que el estrés puede ser desencadenante de diversas patologías en los humanos, como estados depresivos, ansiosos e incluso trastornos adictivos (Chen et al., 2012; Hymel et al., 2014; Sinha, 2008). Los resultados obtenidos con el modelo de derrota social pueden extrapolarse a los humanos en situaciones de estrés psicológico o social a las que estamos expuestos durante gran parte de nuestra vida. Estas situaciones estresantes producen cambios a nivel del eje HHA al igual que ocurre en los animales y alteran la respuesta de los individuos ante las drogas. Nuestros resultados son muy interesantes si pensamos no solo en una diana terapéutica que pueda reducir los cambios neurofisiológicos que produce el estrés y evitar los cambios que se relacionan con el abuso de drogas como el alcohol, sino también, sino también como estrategias preventivas que fomenten el desarrollo de respuestas resilientes a los efectos adversos que induce el estrés social. Además, nuestros resultados proporcionan pruebas conductuales y neurobiológicas de que la exposición a la derrota social durante una etapa temprana de la vida, como la adolescencia, modifica la vulnerabilidad al abuso de sustancias, sensibilizando de forma temprana el sistema inmune. Considerando de vital importancia fomentar y ofrecer mecanismos que favorezcan respuestas de afrontamiento positivo y activo para evitar o reducir las consecuencias negativas que produce el estrés social.

Los resultados que se han presentado en esta Tesis Doctoral pueden ayudar a la identificación de los sujetos más vulnerables a las conductas de tipo depresivo o al consumo de drogas tras la exposición al estrés social. Pero aún más importante, hemos señalado una serie de intervenciones que van a potenciar la respuesta resiliente, ya sea aplicadas durante la adolescencia y previas a experiencias vitales estresantes o durante los episodios de estrés. De esta manera se contribuye al desarrollo de terapias individualizadas con carácter preventivo y terapéutico en el tratamiento de trastornos adictivos.

1. INTRODUCTION

1. General introduction

1. General introduction

Despite stress being a normal and adaptive response, when it is of great intensity or of a chronic nature, marked effects are produced in the organism that lead to different types of disorders (Sandi & Haller, 2015; Selye, 1975a, 1975b). Social conflicts occur frequently both in human society and in the animal world, causing unavoidable stress that worsens the quality of life by inducing different diseases such as anxiety, depression, post-traumatic stress disorder (PTSD) or vulnerability to develop a substance abuse disorder (SUD; Daviu et al., 2019; Sandi & Haller, 2015; Vasconcelos et al., 2015).

The biological stress response allows the organism to act quickly and effectively with its environment. If the stressful situation is transitory, the innate biological mechanisms allow the organism to adapt to the circumstances and provide an adequate response to resolve the situation. If the stressful situation becomes chronic, the organism's own biological response ends up being detrimental to the organism (Sapolsky, 1996; Lijffijt et al., 2014). Different physiological systems of the organism are activated with the purpose of adapting it to internal or external challenges. Among them, the hypothalamic-pituitary-adrenal (HPA) axis must be highlighted, a system that exerts a fundamental role through corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids (GC). The adrenal sympathetic system should also be highlighted, with the stimulation of the production and release of epinephrine and norepinephrine (NE; Kupfermann, 1991). Even so, there are other systems that are activated in the stress response, such as, for example, the immune system (see review by Capuron & Miller, 2011; Costa-Pinto & Palermo-Neto, 2010).

One of the main sources of stress is known as social stress. Social interactions are essential in the development of humans and most animal species. However, some social events can lead to adverse effects such as depression and anxiety, and can even

influence the use of drugs of abuse (Cadet, 2016; Cohen et al., 2016). Emotional and social stressors are the main activators of the stress response in humans, which explains the translational importance of the study of social stress. Animal models can shed light on neurobiological mechanisms underlying the effects of social stress that would be impossible to investigate otherwise. Preclinical research has been studying the mechanisms involved in the stress response and the development of stress-associated mental disorders for decades. In this regard, several paradigms have been developed that model social stress in rodents such as social defeat (SD), social deprivation, social instability, and territorial and maternal aggression. SD is considered the most representative paradigm to study the physiological and behavioral consequences of social stress. SD induces numerous behavioral changes, of which the most studied are the decrease in social interaction (Montagud-Romero et al., 2018; Toth & Neumann, 2013) or the increase in anhedonia (Heshmati & Russo et al., 2015; Riga et al., 2015; Shimamoto et al., 2015), which can be extrapolated and interpreted as indicators of certain pathologies observed in humans, such as depression. In different preclinical studies, it was observed that defeated animals in adulthood may present impaired spatial learning, increased anxiety and altered social behavior (Blanco-Gandía et al., 2019; García-Pardo et al., 2017). The effects of physical or pharmacological stress on synaptic plasticity have been extensively studied, finding that they induce a reduction in cell proliferation and increased cell death, decrease synaptic density and reduce hippocampal volume, either when experienced during adolescence or adulthood (see review Bath et al., 2017; Montes-Rodriguez & Urteaga-Urias, 2018). Changes in dendritic and axonal cytoskeleton morphology induced by SD has been less studied, although there are indications that social stress, through different models (SD, chronic unpredictable mild stress, and maternal separation), produces alterations in dendritic extensions and synaptic alteration (Abdel-Rahman et al., 2004; Barros et al., 2006; García-Gutiérrez et al., 2016; Martin et al., 2017; Yang et al., 2015). Furthermore, SD

induces structural changes in key brain regions in reward behavior, adaptations that induce vulnerability to addiction. Recently, it has been observed that there is a reduction of inhibitory synaptic transmission within the NAc that is critical in the stress response (Heshmati et al., 2020).

Addiction is a chronic and multifactorial disease characterized by out-of-control behavior and relapse in consumption, even after a prolonged period of abstinence (Koob & Volkow, 2016). Among the main risk factors involved in SUD, stress is one of the most important. Stress not only plays a fundamental role in the relapse to drug use, but also in the initiation, escalation and maintenance of the consumption pattern. Stressful experiences modify the activity of brain areas involved in the rewarding effects of drugs (Beutel et al., 2018; Ferrer-Pérez et al., 2018b; García-Pardo et al., 2015; Hwa et al., 2016; Montagud-Romero et al., 2021; Newman et al., 2018; Rodríguez-Arias et., 2016, 2017). Acute stress exposure produces an increase in GC that induces dopamine (DA) activity in the ventral tegmental area (VTA), but when stress becomes chronic there is elevated GC circulation and selective suppression of the ability of the CRH to modulate DA release in the nucleus accumbens (NAc; Douma & de Kloet, 2020). Thus, chronic stress induces a decrease in DA release in limbic and cortical regions (Douma & de Kloet, 2020), with a consequent decrease in pleasurable sensations (Tye et al., 2013).

Alcohol is the most widely consumed substance of abuse worldwide (EDADES, 2019-20; WHO, 2018). The consequences of abusive, chronic and even binge ethanol consumption are diverse and result in a wide range of diseases (cardiovascular, hepatic, cognitive deficits, etc) (Nutt et al., 2010). Preclinical studies have shown that exposure to SD induces an increase in voluntary consumption and motivation to obtain a dose of this substance in adolescent and adult rodents (Favoretto et al., 2020; Karlsson et al., 2017; Macedo et al., 2018; Norman et al., 2015; Rodríguez-Arias et al., 2016). Furthermore, it should be taken

into account that alcohol is considered a "gateway" to the consumption of other substances of abuse and is one of the earliest onset drugs, along with tobacco and hypnosedatives (EDADES, 2019-20). Therefore, the study of the effects induced by stress on the consumption of substances of abuse, such as alcohol in young adolescents is a topic of great interest. Adolescence is a period of great neuroplastic changes in which there is an imbalance between the development of behavioral control and impulsivity, mainly due to the immaturity of the prefrontal cortex (PFC; Sturman & Moghaddam, 2011). It is also a period particularly vulnerable to the negative consequences of stress and associated with the initiation of drug use such as alcohol (Burke & Miczek, 2014; Spear & Swartzwelder, 2014).

In the studies carried out to date, a heterogeneous response occurs in animals subjected to SD. These differentiated responses are also observed in humans. Resilience is defined as the capacity of individuals to maintain adaptive psychological and physical functioning and avoid the onset of mental illness when exposed to high levels of stress (Charney, 2004). The study of resilience is essentially phenomenological, but it is already beginning to identify the psychological and biological characteristics of individuals who are more resilient to psychopathologies, such as depression or PTSD, following exposure to stress (Pfau & Russo, 2015). Most research has focused on the study of resilience to the effects of social withdrawal, anhedonia, or depressive-like behaviors (Krishnan et al., 2007). Numerous changes in different physiological systems have been observed in resilient subjects, such as a less reactive response of the HPA axis, increased neuronal activation, or increased glutamatergic signaling and synaptic connectivity. Differences in dopaminergic transmission have also been observed, mainly characterized by an intrinsic excitability of neurons stimulated by potassium (K^+) channels (Cao et al., 2010; Covington et al., 2010; Christoffel et al., 2012; Linden et al., 2006; Krishnan et al., 2007). Nevertheless, many of the mechanisms involved in the resilient phenotype remain unknown and require further study. This lack of

knowledge is even greater in the study of the resilient response to stress in adolescent animals. Only few studies have observed that there is no homogeneous response to depressive-like behaviors, social avoidance, and anhedonia in adolescents exposed to social stress. That is, in contrast to what is observed in adult rodents, only a small percentage of the animals were fully susceptible or resilient to these behaviors. This highlights the complexity of this stage of the life cycle and the difficulty in interpreting the observed behaviors (Alves-dos-Santos et al., 2020; Vassilev et al., 2021).

Numerous mechanisms are involved in the effects of social stress, but recently numerous studies have focused on the immune system. Social stress influences the organism's immune response, stimulating leukocytes and microglia by increasing peripheral inflammatory cells and brain levels of cytokines, chemokines, and other components of the inflammatory response (Calcia et al., 2016; Rodríguez-Arias et al., 2018). These neuroinflammatory markers can alter the permeability of the blood-brain barrier (BBB), allowing peripheral inflammatory cells to penetrate the central nervous system (CNS), thereby increasing the inflammatory response (Rodríguez-Arias et al., 2017). The release of proinflammatory cytokines and chemokines in turn lead to microglial activation and astrogliosis, causing structural and functional changes in the brain (Calcia et al., 2016). In the last decade, these results have been closely linked to the vulnerability of developing addictive disorders. Therefore, the immune response is a potential mechanism by which increased vulnerability to SUD may be explained. The immune response in the CNS affects DA signaling in the mesolimbic pathway, modifying reward behaviors (Thomas Broome et al., 2020).

A decrease in the neuroinflammatory response has been observed in resilient compared to susceptible animals, results that have also been observed after pharmacological treatments and environmental interventions (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Hodes et al., 2014; Stewart et al., 2015). Several

preclinical studies demonstrate that treatments with non-steroidal anti-inflammatory drugs, such as indomethacin, or hormones such as oxytocin, were capable of buffering and preventing the onset of the neuroinflammatory and behavioral effects of SD (Ferrer-Pérez et al., 2018a, 2021; Giménez-Gómez et al., 2021). In the case of environmental interventions, numerous interventions have been evaluated and there is a large consensus in the scientific literature that moderate physical exercise and environmental enrichment in animal housing can be effective strategies to reduce the neuroinflammatory response, depressive-like behaviors and addiction-related behaviors induced by social stress (Hüttenrauch et al., 2016; Kentner et al., 2018; Lynch et al., 2019; Mul, 2018; Novkovic et al., 2015; Neal et al., 2018; Pietrelli et al., 2018; Salam et al., 2009; Schloesser et al., 2010; Zhang et al., 2018; Zolfaghari et al., 2021). On the other hand, while stress at early ages (childhood and adolescence) can have detrimental long-term consequences in adulthood, some studies show that if this stress is moderate it can induce a more resilient phenotype to future exposures to stressful events. This theory is fostered in the phenomenon of stress inoculation and, although there are few studies to date, it is a very promising intervention to enhance resilience (Dienstbier, 1989; Masten, 2001; Mortimer & Staff, 2004; Rutter, 2006).

Thus, the main objective of the present Doctoral Thesis was to enhance resilience to the negative effects induced by SD on increased alcohol consumption and the increased neuroinflammatory response through pharmacological and environmental interventions prior to SD exposure.

2. Influence of social stress on alcohol abuse

2.1. Social stress conceptualization

Stress can be defined as a non-specific biological response of the organism to a demand formulated from the outside (the environment). It is a normal and adaptive response, in which the organism's reserves are mobilized, energy uptake by the musculature is facilitated, cardiovascular tone is raised to transport oxygen faster and non-essential activities are suspended (Selye, 1975a, 1975b). The effects of stress on the organism are diverse and influence the development of mood disorders, such as anxiety, depression, and SUD.

In general, the terms social, psychological, emotional, sociopsychological or psychosocial stress are used indistinctly to refer to stress generated by components related to social conditions and consist mainly of social support, social organization, socioeconomic aspects, gender, job role, etc., which considerably alter the quality of life. This phenomenon represents a highly topical problem and scientific research has focused on the study of the origin and consequences of socially stressful experiences. Social components can lead to high levels of stress which, in turn, affect health, quality of life and longevity (Molina-Giménez et al., 2008; Sandín, 2002). In addition, variables such as substance abuse, a sedentary lifestyle or poor eating habits can be affected by social stress. This indicates that social stress can induce direct and indirect changes on health (Molina-Giménez et al., 2008; Sandín, 2002). Therefore, animal models of stress are used to study the immediate and long-term effects of social stress on mood disorders and substance abuse.

2.2. How to model social stress in animals: The Social Defeat Model

The SD model is the most widely used animal model in preclinical research and it is ethologically validated. This model is based on the resident/intruder paradigm, which results in social conflict between an intruder individual that is subjected to agonistic encounters when introduced into a cage where another (resident) individual

of the same species already resides (van Erp & Miczek, 1997). This model addresses the consequences of social conflicts between two or more individuals of the same species and allows the study of the strategies that are developed to allow the survival of both, the individual and the group. The interactions that occur during confrontation between conspecifics are influenced by species, age, gender, the individual's previous history, as well as the circumstances in which the confrontation takes place (Blanchard et al., 2001). SD is a more severe stressor than some models of physical stress used in experimental research, as it employs natural, everyday stimuli from social life (Koolhaas et al., 1997), thus resembling natural social situations that occur in group living. It induces long-term physiological and behavioral changes such as those observed in depression and anxiety and may mimic individual differences in the stress response observed in humans (Wang et al., 2021). The SD model can be applied acutely, repeatedly (or intermittently), or chronically, depending on the studied objectives.

Social stress is deeply implicated in neural and behavioral alterations that contribute to the development of mental health disorders and drug addiction (Beutel et al., 2018). Stressful experiences modify the reward system and are implicated in the transition from drug abuse to addiction, leading to increased drug intake and drug-seeking behaviors (Koob & Schulkin, 2019; Miczek et al., 2008; Montagud-Romero et al., 2016, 2018; Ruisoto & Contador, 2019). There is consensus in the literature that SD stress produces significant increases in corticosterone levels (Montagud-Romero et al., 2015; Norman et al., 2015; Niraula et al., 2018; Rodríguez-Arias et al., 2017), modifications in numerous neurotransmitter systems such as serotonergic, dopaminergic or GABAergic (Montagud-Romero et al., 2018), which can produce long-term consequences such as anhedonia, decreased social interaction, anxiety, depression or drug addiction (Burke & Miczek, 2014; Liu et al., 2017; Miczek et al., 2008; Shimamoto et al., 2015).

2.3. Neurobiological response to social defeat

The biological stress response allows the organism to act quickly and effectively in its environment. If the stress situation is momentary, our innate biological mechanisms allow us to adapt to the circumstances and provide the appropriate response to resolve the situation. If the stress situation becomes chronic, the biological response itself ends up being detrimental to the organism (Sapolsky, 1996; Lijffijt et al., 2014). This response can also occur in the absence of actual sensory stimuli. These responses, called anticipatory responses, are generated by a conditioned memory or by species-specific innate predispositions. The anticipatory response consists of a cognitive preparation for an event, involving the interpretation and evaluation of the event. It prepares the organism to cope with the threatening situation by anticipating the arrival of the stressor (Yehuda et al., 1991). The anticipatory response is related to psychological stress, which is exacerbated when no outlets for frustration are seen and when the stressful situation is out of control (Gold et al., 2015).

The main biological mechanism in the stress response is the HPA axis (Koob, 2009, 2010), which is essentially composed of three structures: the hypothalamus, the pituitary and the cortex of the adrenal glands (Perez-Tejada et al., 2013). It is a hormonal mechanism that self-regulates itself through a negative feedback mechanism (Lijffijt et al., 2014). The first link in the HPA axis is CRH in the paraventricular nucleus (PVN) of the hypothalamus (Sarnyai et al., 2001), whose activation in response to a stressful situation triggers its release into the anterior lobe of the pituitary. This connection stimulates the release of ACTH, the second link in the HPA axis. ACTH diffuses through the general circulation until it reaches the adrenal glands where it stimulates the synthesis and secretion of GC, cortisol in humans and corticosterone in rodents (Goeders, 2003; Perez-Tejada et al., 2013). GC sets multiple catabolic processes in motion, which prepare the organism to give a

rapid and effective response to the requirements of the environment. However, by its nature, this response cannot be maintained for a long time, otherwise the organism becomes ill and may even die (Xiong & Krugers, 2015).

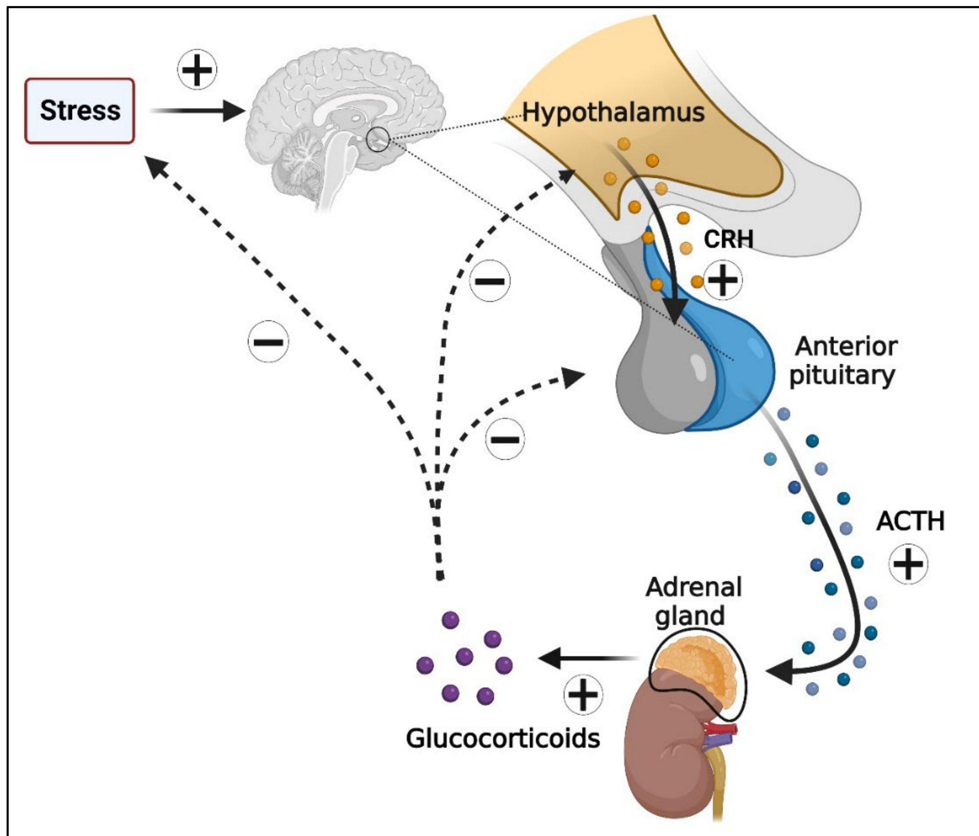

Figure 1. Hypothalamic-pituitary-adrenal axis in the neuroendocrine response to stress. Stress signals trigger the release of CRH from a subset of neurons in the paraventricular nucleus of the hypothalamus. CRH is transported to the adenohypophysis, where it binds to CRH receptors stimulating the synthesis and release of ACTH into the general circulation. In the adrenal cortex, ACTH binds to melanocortin type 2 receptors and stimulates GC secretion into the general circulation. GC inhibits activation of the HPA axis (negative feedback) at the hypothalamic and pituitary levels.

GC in turn gives feedback to the hypothalamus and pituitary (to inhibit CRH and ACTH production) in a negative feedback loop. GC receptors exist at multiple levels within the CNS, essentially in the VTA, NAc and PFC. These receptors are of two types, glucocorticoid (GR) and mineralcorticoid (Cintra et al., 1994). In addition, the CRH factor is also distributed extrahypothalamically in the VTA, NAc and amygdala (Butts & Phillips, 2013; Gold et al., 2015). Two limbic system structures, the hippocampus and the amygdala, and one cortical structure, the medial PFC, are involved in the regulation of HPA axis activity (McKlveen et al., 2015). The hippocampus and PFC are inhibitors of HPA axis activity. In contrast, the amygdala is involved in the activation of the HPA axis (Gold et al., 2015). The amygdala is involved in the perception of fear-eliciting stimuli and the individual's reaction to them.

This functional capacity of the amygdala is given by the type of connections of this structure with other CNS structures. The amygdala receives cortical afferents that provide sensory information and afferents from the PFC that are responsible for controlling emotional responses to stress (Dejean et al., 2015; McEwen et al., 2016). The amygdala is related to other brain structures (PVN, Locus Coeruleus (LC), the parabrachial nucleus, and the raphe nuclei) through the CRH and returns information to the hypothalamus. Thus, decisions and judgments about events and situations can be strongly influenced by emotional state (Dejean et al., 2015; Koob, 2015). It is also involved in the formation of certain types of memories. The activation of the amygdala and corresponding hormonal mechanisms establish memories of frightening situations, which constitute a form of implicit memory, which does not require conscious knowledge. The CRH is crucial in the induction of certain forms of plasticity in the amygdala (Zorrila et al., 2014). Therefore, the PFC and amygdala are structures that play an important role in the emotional component of different processes, such as fear and the anxiety response or memory and learning (Rosenkranz et al., 2003). Other studies show that animals subjected to chronic SD

undergo a down-regulation of DA D2 receptors in the amygdala (Azzinnari et al., 2014; Boyson et al., 2014). In a study of the consequences of two types of social stress (intermittent episodes of SD vs. continuous social stress subordination), it was observed that both caused an increase in cocaine self-administration (SA). In addition, changes in the brain-derived neurotrophic factor (BDNF) and DA were also observed in the VTA-NAc pathway (Miczek et al., 2011). Individual differences in the biological mechanisms of stress, which may have a genetic basis or be acquired throughout life, may determine differences in the vulnerability or predisposition to develop stress-related disorders (Charney, 2004).

Another major region affected by prolonged stress in the organism is the hippocampus, which is involved in learning and memory (Gill & Grace, 2013; Fa et al., 2014). Stressful experiences can affect both hippocampal structure and function, suppressing neurogenesis in adults. For example, chronic SD causes decreased cell proliferation in the dentate gyrus in mice, which may explain some of the stress-related psychopathology (Fa et al., 2014).

2.4. Effects of social defeat on neuronal and synaptic plasticity and morphology

Synaptic plasticity may be the fundamental mechanism through which to respond adaptively to adverse changes in the environment (Montes-Rodríguez & Urteaga-Urías, 2018). The effects of SD on synaptic plasticity have not been extensively studied. Recently, changes in inhibitory synapses have been observed, with reduced inhibitory synaptic transmission within the NAc core being critical in the stress response (Heshmati et al., 2020). However, there are few reports on the changes in neuronal and synaptic morphology induced by some form of social stress. For example, in the study of changes in dendritic extensions, low levels of microtubule-associated protein 2 (MAP-2) have been observed in humans in hippocampal formations of patients with major depression (Soetanto et al., 2010). In preclinical models of chronic SD stress, chronic unpredictable mild stress, and maternal

separation, stressed rodents were observed to undergo decreased MAP-2 gene expression in the hippocampus and cerebral cortex (Abdel-Rahman et al., 2004; García-Gutiérrez et al., 2016; Martin et al., 2017; Yang et al, 2015). This dendritic and synaptic alteration could lead to cognitive alterations (Abdel-Rahman et al., 2004; Martin et al., 2017), influence the development of depressive-like behaviors (García-Gutiérrez et al., 2016; Yang et al., 2015) and vulnerability to ethanol consumption during adolescence (García-Gutiérrez et al., 2016). Likewise, in the prenatal stress model in rats, a decrease in MAP-2 immunostaining levels was observed in the cortex, striatum and hippocampus. This decrease in dendritic arborization could lead to a reduction in neuronal processes and a decrease in the number of synapses (Barros et al., 2006). In relation to the axonal cytoskeleton, there is less scientific evidence in relation to social stress, but a decrease in neuronal filaments 200 kDa (NF200) expression has been observed in mice subjected to maternal separation (García-Gutiérrez et al., 2016). NF200 plays an important role in the stabilization and maturation of pre-existing connections; a dysregulation of its expression can result in an impairment of synaptic connections (García-Gutiérrez et al., 2016).

2.5. Social defeat and alcohol consumption

Ethyl alcohol or ethanol found in spirits is a CNS depressant substance (Chandler et al., 1998; Tabakoff & Hoffman, 2013). It is probably the oldest known drug (Khaderi, 2019) and, when consumed in excess, causes great social harm, such as increased demand for medical care, lower productivity, accidents or crime (Nutt et al., 2010). Alcohol consumption is a causal factor in more than 200 diseases and disorders. It is associated with the risk of developing health problems such as mental and behavioral disorders, including alcoholism, major non-communicable diseases such as liver cirrhosis, some types of cancer and cardiovascular disease, as well as trauma resulting from violence and road traffic accidents (Nutt et al., 2010; WHO,

2018). Worldwide, more than a quarter (26.5%) of all young people aged 15-19 years are current drinkers, amounting to 155 million adolescents (WHO, 2018). Among the Spanish population, alcohol is the most widely consumed substance of abuse. The prevalence in the general population of alcohol consumption in the last 30 days stands at 63% and 8.8% of the surveyed population reports daily consumption, with higher consumption occurring in men than in women (EDADES, 2019-20). Directly or indirectly, alcohol intake can interact with a wide range of neurotransmitters in the nervous system. This interaction occurs due to the fat-soluble nature of ethanol, which allows it to cross the BBB and thus reach the brain. Neurotransmitters and hormones susceptible to interaction with ethyl alcohol are the following: GABA, glutamate, endogenous opioids, DA, epinephrine and NE, acetylcholine, serotonin (5-HT), cannabinoids, CRH, and Neuropeptide Y (NPY; Khaderi, 2019; Tabakoff & Hoffman, 2013; Yang et al., 2022). Acute alcohol consumption produces a facilitation effect on the inhibitory activity of GABA, together with a reduction in the excitatory activity of glutamate, NE and voltage-gated calcium channels (Khaderi, 2019; Tabakoff & Hoffman, 2013). All this results in a decrease of CNS functioning, which in an extreme degree of alcohol intoxication can lead to ethyl coma and death by cardiorespiratory arrest. On the other hand, the acute administration of alcohol produces an increase in the release of DA in the synapses of the ventral striatum (NAc), which has been related to its effect on brain reward and behavioral reinforcement. Subsequently, chronic alcohol administration leads to the progressive development of adaptive changes in these neurotransmitter systems (Khaderi, 2019; Tabakoff & Hoffman, 2013). These are generally compensatory changes to those produced by its acute administration, leading to a state of CNS hyperexcitability, related to noradrenergic, glutamatergic and voltage-gated calcium channels hyperfunction, together with GABAergic hypofunction and a state of hypodopaminergia, which may be expressed clinically in the form of alcohol withdrawal symptoms (Khaderi, 2019; Tabakoff & Hoffman, 2013).

Alcohol dependence is characterized by deficits in the physiological regulation capacity of endogenous motivation and reward systems. Several brain structures are postulated to be responsible for these effects that impact behavior, such as the limbic system, the amygdala, the hippocampus, the caudate nucleus, the NAc and the frontal lobe. Dysfunction of the aforementioned systems is behind phenomena linked to alcoholism such as dependence, alcohol intoxication or withdrawal syndrome. Alcohol-dependent patients may continue to exhibit withdrawal symptoms such as anxiety, insomnia, irritability, anhedonia, dysphoria, craving, impulse desregulation and other symptoms for weeks or months after withdrawal from this drug (Guardia et al., 2011).

It is now well known that stress can facilitate the use of drugs of abuse, such as alcohol, and favor the development of addiction. Alcohol can have a powerful reinforcing effect, given that, in addition to its positive reinforcing effect (due to the activation of the brain reward circuit), it also relieves unpleasant emotional states or withdrawal symptoms. For this reason, people suffering from symptoms of anxiety, worry, anguish, phobias, insecurity, obsessions, PTSD, sleep disorders, irritability, guilt, depression and other mood disorders can obtain an intense reinforcing effect from alcohol consumption (Guardia et al., 2011).

As already mentioned, stress affects DA, which is the main neurotransmitter related to pleasure sensations (Belujon & Grace, 2015; Goeders, 2003; Koob & Schulkin, 2019). A situation of moderate and transient acute stress will increase DA release in the reward pathways, which project from the VTA to the NAc and the PFC. This increased DA can induce a sense of well-being in moderate stress situations (Belujon & Grace, 2015). In contrast, exposure to chronic stress decreases DA system activity and with it, feelings of pleasure (Tye et al., 2013; Deal et al., 2018). This interaction between stress mechanisms and the DA system favors the development of addiction. Acute stress causes increased DA release and chronic stress is a source of anxiety

and lack of pleasure, both situations that favor consumption, albeit through antagonistic mechanisms (Sinha, 2008; Tye et al., 2013). The VTA is a critical component in the mesocorticolimbic circuit in which CRH release can cause synaptic neuroadaptations of DA neurons within the mesolimbic pathway (Boyson et al., 2014).

On the other hand, the activation of the brain's stress systems has been related to the negative emotional state produced by substance dependence, which directs behavior to the consumption of the drug by negative reinforcement (alleviating the discomfort caused by the absence of the substance). The extended amygdala is the circuit that links the stress and reward systems (Koob & Volkow, 2016). This is composed of the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CeA) and the NAc shell (Koob & Volkow, 2016). Moreover, CRH, NE and DA are involved in this circuit (Koob, 2010). The extended amygdala is innervated by the noradrenergic pathway originating from the LC (Aston-Jones et al., 1996) and its main projections are directed to the hypothalamus and brainstem (Koob & Volkow, 2016). The midbrain, including the VTA, sends DA projections to the BNST and CeA (Hasue & Shammah-Lagnado, 2002). Both DA and noradrenergic activity affect CRH transmission in the CeA and BNST. CRH neurons project from the CeA to the BNST and VTA and in turn, from the BNST to the VTA (Rodaros et al., 2007). CRH action in the VTA results in stimulation of DA and glutamate release (Polter & Kauer, 2014).

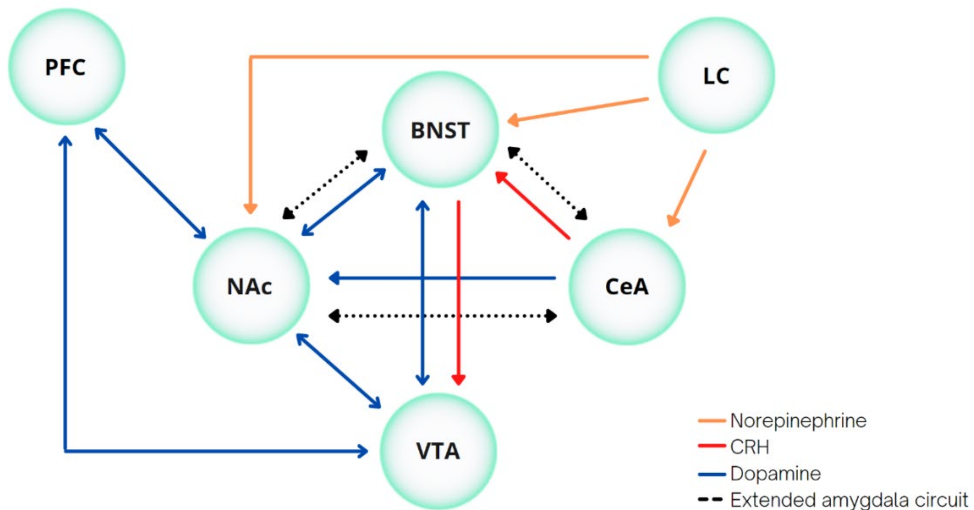

Figure 2. Relationship between brain systems mediating addictive behavior and social stress. The LC is the most important noradrenergic neuronal area of the CNS, implicated in many of the sympathetic effects caused by stress due to increased NE production. In turn, this increase in NE by the LC is related to the activation of brain areas drug-sensitive brain areas, such as the extended circuit of the amygdala, formed by the CeA, the NAc and the BNST. Neurotransmitters such as NE, CRH and DA interact in this circuit, in which areas such as the PFC and VTA also participate.

Numerous preclinical studies support that rodents exposed to SD stress increase their consumption, preference and motivation for alcohol, having been evaluated with different paradigms. In addition, several studies show that social stress produces increased sensitivity to the conditioned rewarding effects of ethanol, delays the extinction of memories associated with ethanol consumption, and induces the reinstatement of place preference associated with consumption in the conditioned place preference (CPP) paradigm (Karlsson et al., 2017; Macedo et al., 2018). In the study of voluntary ethanol intake, escalation in consumption has been observed even after 10 days since the last exposure to SD (Croft et al., 2005; Deal et al., 2018; Caruso et al., 2018; Norman et al., 2015; Hwa et al., 2016; Karlsson et al. al., 2017; Newman et al., 2018). In particular, in oral ethanol SA, significant increases in

consumption, a greater number of active responses, and increased motivation to obtain the substance in the long term have been described in defeated rodents (Norman et al., 2015; Rodriguez-Arias et al., 2016; Van Erp & Miczek, 2001; Riga et al., 2014; Montagud-Romero 2021).

This increase in alcohol consumption induced by social stress could be due to stress-induced neuroadaptations, which ultimately produce changes in hypothalamic, extrahypothalamic and mesocorticolimbic circuits, which are related to stress and reward (Holly et al., 2016; Hwa et al., 2016; Newman et al., 2018). Both systems (addiction and stress) are closely related and any type of environmental stress could affect the brain reward system (Belujon & Grace, 2015; Koob & Schulkin, 2019; Yap & Miczek, 2008).

3. The adolescent brain

3.1. Maturation of the adolescent brain

Adolescence in humans is considered to begin with the onset of biological changes associated with puberty (sexual maturity), and ends when the individual assumes adult social roles and sleep patterns are regularized (Abbott, 2005). This period is usually divided into three categories: early adolescence between 10-14 years, late adolescence between 15-19 years, and young adulthood between 20-24 years (Patton et al., 2016). In rodents, the term "adolescence" comprises the entire postnatal period from weaning (postnatal day (PND) 21) to adulthood (PND 60). It is divided into three distinct periods: early adolescence (prepuberty, PND 21-34), middle adolescence (peri-adolescence, PND 34-46) and late adolescence (young adulthood, PND 46-59; Bates & Trujillo, 2021; Zoratto et al., 2018).

Adolescence is a critical period of brain maturation characterized by multiple cognitive, behavioral, and biological changes related to important changes in emotional and cognitive functions (Pickles et al., 1998, Spear, 2000; Bava & Tapert, 2010). This period is characterized by behaviors common to all species, such as hyperphagia, delayed circadian cycle, increased social interactions, increased novelty seeking and risk-taking behaviors (Spear, 2000). The activation of steroid hormones during adolescence triggers numerous organizational effects, resulting in structural changes, such as myelination, apoptosis, neuronal pruning and remodeling of dendritic spines, thus determining the adult behavioral response to steroids or sensory stimuli (Vigil et al., 2011). Brain maturation in adolescence affects the whole brain and involves a significant loss of gray matter and number of synapses and synaptic receptors, mainly in the neocortex, as well as an increase in white matter related to increased myelination of interhemispheric and cortico-subcortical connections (Gogtay et al., 2004). The acquisition of mature cognitive skills in the domains of decision-making, behavioral inhibition, and working memory is attributed to the functional maturation of the PFC during adolescence. However,

during adolescence, a decrease in major neurotransmitter receptors is experienced. Primarily there is excitatory synaptic pruning, i.e., there is a decrease in NMDA receptors and in the extent of excitatory glutaminergic stimulation to the cortex. In contrast, GABAergic inhibition is greater during adolescence due to pre- and postsynaptic changes, and the number of GABA synapses increases (Caballero et al., 2016; Zimmermann et al., 2019). In the limbic regions, there are also important changes, with reductions in gray matter in the hippocampus, the striatum and other subcortical structures, leading to increases in white matter in cortical and subcortical fiber tracts, and in the connections between the amygdala and the striatum to the PFC (Caballero et al., 2016; Zimmermann et al., 2019). In addition, there is a decrease in glutamate receptors in the hippocampus and DA receptors in the striatum (Zimmermann et al., 2019). The PFC requires a longer time to reach maturity compared to limbic regions during adolescence, resulting in a lack of synchrony between the frontal area and cortico-subcortical regions. This leads to an elevated activation in mesocorticolimbic regions involved in reinforcing behavior and attenuated sensitivity to aversive stimuli, resulting in novelty-seeking behavior with risky behaviors and increased emotional reactivity (Sturman & Moghaddan, 2011).

Due to the major neurobiological changes that occur during adolescence, it is generally considered a time of increased vulnerability to various mental disorders, such as addiction and mood disorders that often first appear during adolescence (Lo Iacono & Carola, 2018).

3.2. Adolescence and stress response

Exposure to social stress during adolescence causes disruption of normal neural development and increases the risk of mood disorders such as depression and anxiety in adulthood (Burke et al., 2017; Iñiguez et al., 2014). Although adolescents show greater ACTH and corticosterone release in response to an acute stressor than adults (Bingham et al., 2011; Mancha-Gutiérrez et al., 2021), their response to SD evolves

as repeated encounters occur. Adolescent mice do not differentiate between play-fighting and SD encounters. The experience of defeat during adolescence affects play behavior, with defeated rodents adopting more submissive postures during play fights (Buwalda et al., 2013; García-Pardo et al., 2014; Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017). Changes in the HPA axis and hormonal changes promoted by puberty (e.g., an increase in testosterone levels) may underlie the progression in SD experience that takes place during adolescence (Romeo, 2017; Wright et al., 2012).

As in defeated adult rodents, several studies have reported an increase in anxiety and depressive-like behaviors in socially defeated adolescent rodents (Huang et al., 2013; Iñiguez et al., 2014; Mancha-Gutiérrez et al., 2021; Watt et al., 2009). These behaviors are characterized by a significant decrease in social exploration and an increase in avoidance, flight, defense and submission behaviors in the social interaction test (Rodríguez-Arias et al., 1998). In addition, transcriptional changes underlying molecular and behavioral changes that influence the development of depressive symptomatology have been observed, such as reduced levels of total BDNF and isoform IV in PFC (Xu et al., 2017). These results suggest that epigenetic changes in the BDNF gene in the mPFC after SD in adolescence may be involved in the regulation of depression-related cognitive dysfunction and antidepressant action in adulthood. Decreased levels of Pitx3 transcription factors have also been observed in the VTA, and an increased expression of the TrkB receptor (Montagud-Romero et al., 2017).

Likewise, exposure to SD during adolescence influences the development of vulnerability to the consumption of substances of abuse such as alcohol. Preclinical studies have observed that socially defeated adolescent rodents consume more alcohol and show increased drug motivation even three weeks after the last SD experience (Rodríguez-Arias et al., 2016). Although the initial

SD experience is different in adolescent animals, there is an increase in the rewarding effects of alcohol regardless of the age at which stress exposure occurs (Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017). These results confirm that social stress in this developmental period has lasting consequences in adulthood (Burke et al., 2011; Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017).

3.3. Adolescence and reward system

During adolescence, impulses generated by limbic system brain structures cannot be adequately inhibited by the PFC regions (Casey et al., 2008). Therefore, behaviors with high risk components and high levels of stress appear at this stage (Bava & Tapert, 2010; Sturman & Moghaddam, 2011). This peculiarity becomes a challenge when regulating behavior in adolescence, since most emotional impulses do not find a "barrier" that can contain the emotional intensity that motivates the behavior. Thus, adolescence is characterized by a diminished, self-regulatory capacity as a consequence of limited inhibitory capacity, poor control regulation, hyperactivity of the amygdala, and hyperactivity of the DA in the NAc when processing appetitive stimuli (Rodríguez-Arias & Aguilar, 2012). Additionally, adolescence is considered a particularly stressful time due to the great changes that occur, and it is very common for individuals to perceive stressful situations with greater intensity and feel overloaded. When this occurs, poorly managed stress can lead to emotional instability, anxiety, withdrawal, aggressiveness, physical illness, or inappropriate coping skills, such as the use of drugs like alcohol (Buchanan et al., 1992).

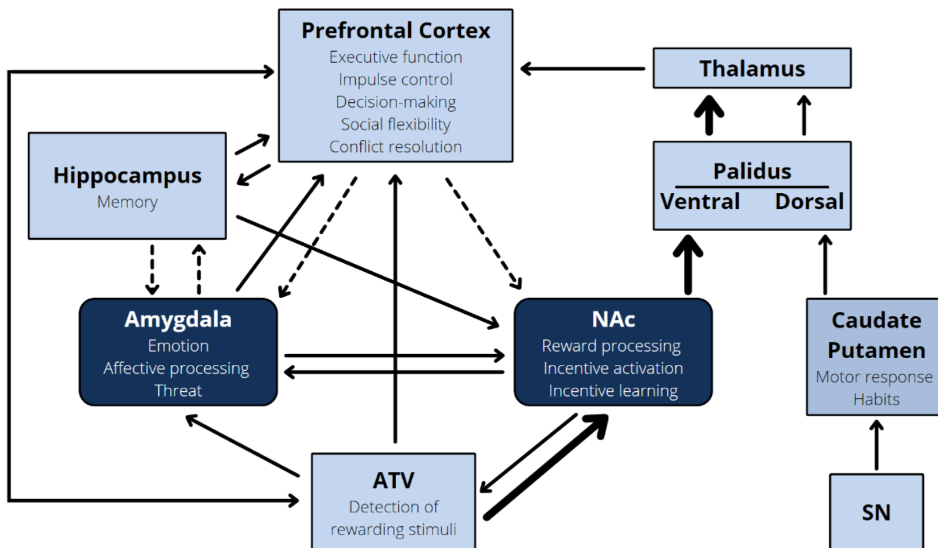

Figure 3. Neural circuitry involved in motivated behavior during adolescence. The thick lines represent hyperactive areas or connections and the faint dashed lines represent more hypoactive brain areas in adolescent individuals than in adults.

It has often been described that behavioral sensitivity to rewards peaks during adolescence and then gradually declines during adulthood (Steinberg et al., 2009; Steinberg 2010). Sensitivity to a basic reward is greatest during early adolescence (Spear, 2013). During this stage, there is a temporary decrease in the efficacy of mesolimbic DA projections and a lower basal rate of DA release with a higher uptake and lower DA release compared to adults (Stamford, 1989). This lower activation would lead adolescents to seek greater sensations and rewards and engage in riskier behaviors in an attempt to compensate for the dopaminergic deficit. The reward sensitivity and elevated risk-taking experienced by adolescents encourages early experimentation with drugs because they are generally more rewarding and less aversive (Schramm-Sapyta et al., 2009). Drug exposure and use during adolescence often predict a greater likelihood for continued use in adulthood (Merline et al., 2004; Spear, 2015). Alcohol is the first-choice drug among adolescents. Clinical and preclinical studies confirm that excessive alcohol consumption during this period

sensitizes brain regions and developmental processes that are implicated in drug-taking behaviors (Guerri & Pascual, 2010) and produces negative short- and long-term consequences, such as memory impairment and neuronal cell death in several brain regions, which are largely irreversible (Guerri & Pascual, 2010; Pascual et al., 2018). Exposure to drugs of abuse can induce neurobehavioral, neurochemical and neuroendocrine effects in the adolescent rat brain, thus affecting the growth process and systems involved in plasticity and cognition (Jain & Balhara, 2010).

4. Resilience to social stress

4.1. Resilience conceptualization

Stress is one of the main risk factors in the development of mental disorders. However, there are some individual traits or internal conditions that can be activated in the face of threats or stressful situations that condition the type of coping expressed in response to the stressor (Dantzer et al., 2018; Snyder et al., 2015; Wood & Bhatnagar, 2015). As a result, some individuals are more vulnerable than others to develop stress-related diseases, as this partially depends on the cognitive strategies used to solve problems and the ability to develop resistance or protective strategies that are linked to individual variables. Resilience is understood as "the capacity to recover, overcome and adapt successfully in the face of adversity, and to develop social, academic and vocational competencies despite being exposed to severe stress or simply to the tensions inherent in today's world" (Henderson & Milstein, 2003). This concept enables the understanding to accompany human beings of all ages in their life conflicts and allows them to face the possible adversities of life. There are two elements that constitute this term: one that highlights the adverse context and another that gives strength to how this context is faced. In the first case, it is expressed in terms that include problems and adversities, risk factors, biological risk factors or stressful life events, and severely stressful and cumulative life circumstances. In the second case, it is expressed in terms of coping, as the ability of the human being to cope, the ability to protect one's integrity, a conjunction between environmental factors and temperament, and a dynamic process resulting in positive adaptation (Henderson & Milstein, 2003).

In recent years, there has been increasing interest in characterizing the resilient response to the adverse effects of social stress. However, the neurobiological mechanisms underlying the resilient response are not yet known. To date, we know that the effects of stress on the HPA axis depend on the timing, magnitude, type and duration of stress. In clinical studies of patients with major depression and PTSD,

elevated blood glucocorticoid levels have been reported in only approximately 33% of patients (Stetler & Miller, 2011). Among a small subset of depressed patients, reduced glucocorticoid levels have been observed, correlating with less severe symptoms. Despite observing these differences between patients, the mechanisms involved with the HPA axis and a resilient response to stress are currently unknown. Another component that has been linked to resilience in humans is dehydroepiandrosterone. This is a precursor of anabolic steroid synthesis, which is released in the stress response along with cortisol and can act on steroid receptors. Several studies have associated elevated dehydroepiandrosterone levels with less severe symptomatology and better coping in some PTSD patients (Rasmusson et al., 2003; Yehuda, 2006). On the other hand, testosterone is related to social rank, feelings of success, dominance and aggressiveness (Oliveira et al., 2009). Clinical studies show that, following exposure to stress, testosterone levels in blood and saliva decrease, and are often lower in patients diagnosed with major depression or PTSD (Mulchahey et al., 2001; Pope et al., 2003). It has been suggested that testosterone may be a factor that promotes positive mood states and social connectedness. It has also been observed that the peptide neurotransmitter NPY may act as a protective factor against stress. Elevated blood levels of NPY during highly stressful situations predicted lower states of psychological distress and fewer dissociative symptoms in the military (Morgan et al., 2002). As we will see below, to contribute to the study of the mechanisms underlying resilience, animal models have been used as a key tool to define the neural circuits and molecular adaptations that contribute to this phenomenon.

4.2. Characterization of the resilient phenotype in murine models

As we have discussed in the previous sections, passive coping strategies or responses during SD exposure are linked to social stress-induced maladaptive behaviors in rodents (Ballestín et al., 2021; Hawley et al., 2010; Russo et al., 2012; Wood &

Bhatnagar, 2015). Animals exhibiting passive coping mechanisms appear to be susceptible to physiological dysfunction and mental disorders (Hawley et al., 2010; Murrough & Russo, 2019; Russo et al., 2012; Wood & Bhatnagar, 2015). However, not all animals exposed to social stress develop depression-like, anxious, or addictive behaviors. As in humans, a subset of mice exposed to SD will be susceptible to these effects and will develop major disorders such as social avoidance, anhedonia, anxiety, depressive-like behaviors, and increased drug use (Krishnan et al., 2007). However, some rodents will be resilient to these consequences and will be able to cope adaptively with stress (Cathomas et al., 2019).

Resilience to SD stress in rodents is usually defined as the absence of social avoidance, anhedonia and metabolic syndrome, characterized by obesity, overeating or central leptin resistance (Berton et al., 2006; Golden et al., 2011; Krishnan et al., 2007; Lutter et al., 2008), although factors such as genetic differences between rodent strains have to be taken into account. While around 35-45% resilience to SD stress is observed in the C57BL/6 mouse strain and Wistar rats, higher percentages of resilience are observed in CD1 mouse strains and Sprague Dawley rats (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Golden et al., 2011; Vidal et al., 2011). These differences will also depend on the intensity and duration of SD, as well as the influence of other stressors such as restraint or chronic mild stress, or the role of complex genetics in the regulation of risk and resilience. Preclinical studies show that at least 35% of these animals do not exhibit increased HPA axis reactivity induced by SD exposure or behaviors such as social avoidance, hyperthermia, anhedonia, metabolic syndrome, or development of vulnerability to substance abuse (for a review, see Cathomas et al., 2019; Murrough & Russo, 2012; Russo et al., 2012). This does not mean that they do not develop symptoms; they also experience maladaptive behavioral changes but develop resilience to most SD-induced negative consequences. Resilience to stress arises primarily from active coping strategies, both behavioral and molecular. In the scientific literature, it has been hypothesized

that strategies that limit stressful experiences can promote resilience (Christoffel et al., 2012). In the SD model, it has been observed that rodents that adopt less submissive postures during resident attacks subsequently show less social avoidance. This suggests that this behavioral strategy may diminish the effects of social stress (Ballestín et al., 2021; Wood et al., 2010). In recent years, the resilient response has been further characterized, not only at the behavioral level, but also by delving into the neurobiological mechanisms involved that differ from passive responses. The main findings related to the resilient phenotype will be discussed below.

As mentioned above, the HPA axis plays a key role in mediating stress responses associated with depressive and anxiety behaviors in humans and animal models. Although little is known about the HPA axis adaptations that contribute to resilience, epigenetic mechanisms favoring active coping with stress have been observed. Following exposure to chronic SD, increased Crh gene expression was observed in the PVN of the hypothalamus of susceptible rodents that induces the development of social avoidance (Elliot et al., 2010). In contrast, in SD-resilient rodents, hypermethylation and silencing of the Crh gene was observed, with a consequent decrease in CRH expression, preventing the development of social avoidance. Other studies have shown that resilient mice did not develop increased corticosterone levels or adrenal hypertrophy, as observed in mice susceptible to SD-induced depressive-like behaviors. Furthermore, lower hippocampal GR expression was observed in susceptible mice compared to resilient rodents, and resilient rats show lower CRH efficacy (Dedic et al., 2019; Moncek et al., 2004). Thus, increased numbers of GRs in brain cells and decreased levels of circulating GC in resilient animals indicate adequate function and cessation of the HPA axis in the face of recurrent stressful stimuli (Dedic et al., 2019; Moncek et al., 2004).

In relation to glutamatergic signaling and synaptic connectivity, clinical studies postulate that depression and anxiety are due in part to hypoactivation and a reduced

volume of some frontal cortical regions and the hippocampus, which controls subcortical structures such as the NAc and amygdala (Duman, 2014; Yan & Rein, 2022). Preclinical studies have observed increased neuronal activation, observing increased expression of c-Fos, FosB or ΔFosB in glutamatergic neurons of the PFC in chronic SD-resilient mice, which could be a positive adaptive factor in the face of social stress (Adamec et al., 2012; Covington et al., 2010; Lehmann & Herkenham 2011). Furthermore, by direct optogenetic stimulation of mPFC neurons with the rhodopsin channel and glutamatergic microcircuitry from the PFC to the NAc, an antidepressant effect promoting resilience to SD has been obtained (Christoffel et al., 2012). Scientific evidence supports the hypothesis that resilience develops through active adaptation opposite to the mechanism that produces susceptibility (Christoffel et a., 2011; Vialou et al., 2010). It appears that resilience is promoted by an increased induction of ΔFosB in NAc medium spiny neurons, which induces GluA2 (GluR2) expression. This subunit of the glutamate AMPA receptor reduces Ca2+ permeability and global conductance of AMPA channels (Vialou et al., 2010). These results are in agreement with results observed in depressed humans examined postmortem, where decreases in ΔFosB and GluA2 levels have been observed in the NAc (Vialou et al., 2010). In the same context, the resilient phenotype has also been associated with a normalized response of the dopaminergic system, with a normal activation rate of VTA dopaminergic neurons after SD exposure (Krishnan et al., 2007). Increased activation of VTA DA neurons in mice susceptible to the effects of SD has been associated with increased social avoidance and deficits in sucrose preference. This hyperexcitability of DA neurons in the VTA in susceptible mice is in part mediated by the induction of hyperpolarization-activated cation current, which increases the intrinsic excitability of these neurons. In resilient mice, the increase in this cation current also occurs, but large increases have been observed in a group of K+ channel subunits, which are not observed in susceptible animals. This induction of K+ channels prevents the increase in hyperpolarizing current, thus

promoting resilient behavior (Cao et al., 2010; Krishnan et al., 2007). By optogenetic or pharmacological treatments, this additional increase in hyperactivity of VTA DA neurons in susceptible mice can be induced to produce homeostatic plasticity and reverse depressive-like behaviors (Chaudhury et al., 2013; Friedman et al., 2014). Although further studies are needed, K+ channels appear to be pivotal in the development of a resilient response.

Although there are few studies evaluating the role of GABA in the resilience to the negative effects of SD, it has been observed that chronic SD activates GABA neurons of the dorsal raphe nucleus and strengthens the inhibition of 5-HT neurons in susceptible mice. In contrast, this effect has not been observed in mice resilient to depressive-like behaviors. By optogenetic inhibition of GABA neurons in the dorsal raphe nucleus, disinhibition of 5-HT neurons and, consequently, an increase in resilience has been observed (Challis et al., 2013). Other effects of SD in susceptible animals that have not been observed in resilient animals include reduced levels of inhibitory synaptic markers and protein expression in the NAc (Heshmati et al., 2020) and down-regulation of GABAB receptors in the habenula with elevated c-Fos expression (Li et al., 2021). In studies with knockout (KO) mice, it has been observed that GABAB(1a) KO mice are susceptible, and GABAB(1b) KO mice are resilient to SD-induced social avoidance behaviors (O'Leary et al., 2014). Moreover, LC NE neurons have direct connections within the VTA and also appear to regulate SD vulnerability through the inhibitory control of VTA DA neurons (Isingrini et al., 2016). LC NE neurons projecting to the VTA exhibit enhanced activation in resilient, but not susceptible mice, and optogenetic activation of LC neurons in susceptible mice reverses depressive-like behaviors (Zhang et al., 2019).

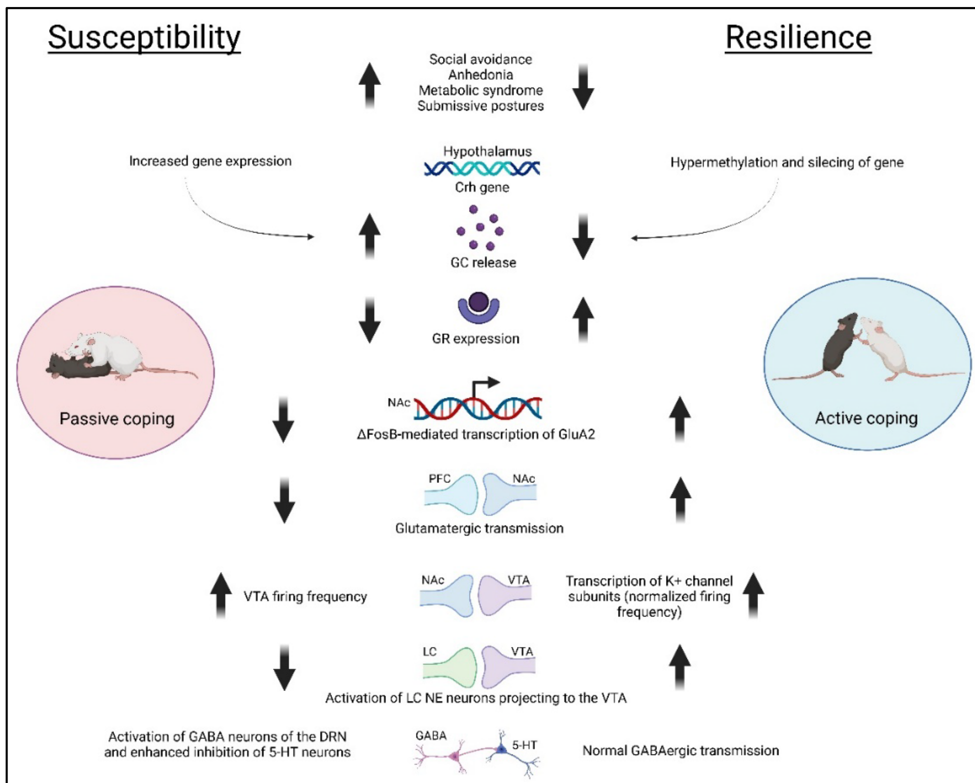

Figure 4. Characterization of susceptible/resilient phenotypes in mice.

In relation to the characteristics of the resilient phenotype during adolescence, it should be noted that it has not been widely studied and the results are not conclusive. Vassilev et al. (2021) observed a higher proportion of resilient mice in the social interaction test (SIT) and increased risk behavior after exposure to a model of SD repeated twice daily for 4 days during adolescence. In addition, they observed an overall increase in the volume occupied by DA fibers in the prelimbic (PrL) and infralimbic cortex in resilient mice, but not in susceptible mice. This indicates changes in DA connectivity in the PFC. Despite these results, they observed that adolescent mice both susceptible and resilient to depressive-like behaviors showed impaired inhibitory control during adulthood, and greater risk-taking behavior in the case of resilient mice. Both impaired inhibitory control and high risk-taking are traits

associated with vulnerability to substance abuse (Egervari et al., 2018). In another study using chronic SD in adolescent mice, it was observed that only 20% of the mice showed a fully susceptible phenotype (increased anhedonia and increased social avoidance) and 30% were fully resilient. While none of the phenotypes showed anxious-like behaviors, it was observed that resilient mice showed reduced weight gain and increased corticosterone levels 24h after chronic SD (Alves-dos-Santos et al., 2020). These results show that, contrary to what has been previously observed in adult mice, the response to social stress due to SD in adolescents is more complex and peculiar. This topic should be further explored for a better understanding of the mechanisms underlying stress resilience at this stage of the life cycle. Conversely to these results in adolescents, several studies demonstrate that mice resilient to the effects of SD stress during adulthood show a consistent phenotype. These mice are resilient to the depressive-like behaviors produced by SD, and are also resilient to increased consumption of substances of abuse such as cocaine or alcohol (Ballestín et al., 2021; Giménez-Gómez et al., 2021, Krishnan et al., 2007; Riga et al., 2020).

4.3. Resilience to stress as a protective factor against the development of an addictive disorder

As discussed in Section 2.4, SD induces vulnerability to the development of addictive disorders such as alcohol use disorder (AUD). Although there is extensive literature supporting the impact on resilience and vulnerability to drug use, such as alcohol or cocaine, of different models that induce social stress, only few papers have studied resilience to the SD-induced increased reinforcing properties of drugs of abuse. Krishnan's (2007) study was a pioneer in characterizing the resilient/susceptible phenotypes according to the response to social stress in rodents. The resilient phenotype was characterized by the lack of display of anhedonia, social avoidance, and anxiety-like behavior exhibited by susceptible mice. In addition, differences in the conditioned rewarding effects of cocaine were observed. In

contrast to susceptible mice, resilient mice did not exhibit CPP induced by a subthreshold dose of cocaine. Other studies corroborate these results obtained in cocaine CPP (Ballestín et al., 2021; Calpe-López et al., 2020; Giménez-Gómez et al., 2021; Ródenas-González et al., 2021).

In relation to alcohol, only two studies have evaluated resilience to social stress on alcohol consumption. Riga et al. (2020) were the first to suggest that resilience to depressive-like behaviors may protect against the development of AUD-like phenotypes in rats. In their study, rats classified as depression-prone were more vulnerable to alcohol, emulating patterns of alcohol dependence as observed in individuals with an AUD. In this study, animals were exposed to repeated SD and subsequently isolated for several weeks. Their depression profile was assessed during isolation, weeks after the last defeat. In addition to social avoidance, cognitive performance was also used to classify the animals into resilient or susceptible to depressive-like behaviors. Although the authors stated that animals prone to depression showed a more intense drinking pattern, their increase in alcohol consumption during SA acquisition was not significantly greater. However, they observed a greater response to alcohol reward during fixed ratio 3 (FR3) and progressive ratio (PR). In addition to a high motivation toward alcohol, these depression-prone rats showed a tendency toward extinction resistance and relapse facilitation. Similarly, among rats exposed to SD stress, there was a subpopulation in which SD exposure increased anxiety-like behavior and induced SA alcohol escalation. Compared to resilient rats, vulnerable rats showed a strong positive up-regulation of vasopressin and oxytocin that was positively correlated with the magnitude of anxiety-like behavior and alcohol SA (Barchiesi et al., 2021). These studies suggest that a propensity for depression or anxiety increases vulnerability to AUD, whereas resilience to stress-induced mental disorders may protect the individual from developing an AUD. All of these studies suggest that differences in

the ability to cope with stressful situations or in the stress response result in different tendencies to develop addictive behaviors. It should be emphasized that these studies have been conducted in adult rodents, although research has begun to investigate the resilient phenotype in adolescents subjected to SD (Alves-dos-Santos., 2020; Vassilev et al., 2021) but it has not yet been characterized in relation to the increase in drug use induced by SD.

5. The role of the immune system in the development of addiction

5.1. Overview of the immune system

The immune system is the physiological tool formed by a complex network of cells, tissues, organs and the substances that they produce, which help the organism to fight infections and other diseases. In addition, it is a self-defense system that also recognizes oncoproteins and eliminates neoplastic cells on a daily basis (Masi et al., 2017; Parkin & Cohen, 2001). The immune response is defined as the global and coordinated response of all these cells and molecules against microorganisms and immunosurveillance against the occurrence of tumors, infections, and autoimmune and allergic diseases (Masi et al., 2017; Parkin & Cohen, 2001).

The immune system is composed of two functional units: the innate immune system (non-specific) and the adaptive immune system (highly specific). Both interact with each other to preserve the homeostasis of the organism (Parkin & Cohen, 2001). The innate immune system is our body's first line of defense and has pre-existing mechanisms that activate quickly and provide the adaptive system with time to activate, expand, and select the best defense (Nicholson, 2016). The innate immune system responds in the same way to different infectious stimuli and has limited specificity. It is mainly composed of physical and chemical barriers (epithelia, enzymes), phagocytic cells (neutrophils, macrophages), natural killer cells, the complement system, cytokines, and Toll-like receptors (TLRs). External barriers (physical, chemical and biological) constantly act to prevent the entry of microorganisms into the organism and their proliferation. If the foreign agent overcomes these barriers, internal innate mechanisms (soluble factors and cellular components) are activated to try to prevent its establishment, development and pathogenic action (Fraser et al., 2004; Mogensen, 2009). Threat detection is carried out by pattern recognition receptors (PRRs). PRRs are encoded in the germline and monitor the extra- and intracellular space. When activated, they initiate signal transduction that promotes antimicrobial and proinflammatory functions of the cells

expressing them. The expression of PRRs can also occur in epithelial cells that form the BBB. PRRs primarily recognize conserved microbial molecules called pathogen-associated molecular patterns (PAMPs), but they can also identify danger or damage signals from their own cells that are expressed or released in response to stress, tissue damage and/or cell death by necrosis (Fraser et al., 2004; Mogensen, 2009). These endogenous molecules are termed damage-associated molecular patterns (DAMPs). Structurally, DAMPs can be lipids, carbohydrates, proteins, lipoproteins/glycoproteins or microbial nucleic acids, as detailed below. DAMPs can be produced as a result of cellular damage caused by infections, but can also indicate sterile injury to cells caused by some other reason, such as chemical toxins, burns, trauma, or reduced blood supply (Roh & Sohn, 2018). PRRs can be grouped into two main kinds, the first of which consists of TLRs and C-type lectin receptors, which are anchored on the plasma membrane or in endocytic compartments. These receptors detect the presence of microbial ligands in the extracellular space and in endosomes. Nucleotide-binding oligomerization domain receptors, retinoic acid-inducible gene I-like receptors and cytosolic DNA sensors form the second group and are localized in the cytoplasm, where they detect the presence of intracellular pathogens (Roh & Sohn, 2018). In addition, PRR-induced signal transduction leads to the production of proinflammatory cytokines and interferons (IFNs). The activation of PRRs also initiates a non-transcriptional response, such as induction of phagocytosis, autophagy, cell death and cytokine processing. Coordination of these signaling pathways orchestrates the immune response from initial infection control to triggering an appropriate adaptive immune response (Roh & Sohn, 2018).

When an infection cannot be controlled through the innate immune response, a defense mechanism more adapted to the aggressor, the adaptive immune response, will be activated. The adaptive or acquired immune system develops as we are exposed to pathogens and other potentially harmful substances throughout our lives. The cells involved in the adaptive immune response are T lymphocytes, which act

as signaling cells, B lymphocytes, as secretors of immunoglobulins or antibodies, and antigen-presenting cells (APCs), which activate T lymphocytes and may include macrophages, dendritic cells, B lymphocytes and, in the case of the CNS, microglia cells (Fraser et al., 2004). Adaptive immune responses are composed exclusively of antigen-specific reactions mediated by T lymphocytes and B lymphocytes, and are unique to higher animals. Lymphocytes neutralize pathogens with antibodies, activating macrophages or preventing the destruction of their own cells. Their specialization takes place in the primary lymphoid organs (bone marrow and thymus), where their receptors are expressed and the most suitable ones are selected. T and B lymphocytes have specific receptors for each antigen whose genetic information is replicated and mixed in each new generation of these cells. Adaptive immunity derived from T lymphocytes is called cell-mediated immunity and that derived from B lymphocytes is called humoral immunity (Fraser et al., 2004). Cell-mediated immunity is the main defense mechanism against intracellular microorganisms (viruses and some bacteria). T lymphocytes are divided into two main subpopulations: CD4+ T lymphocytes, whose main function is the secretion of cytokines, and CD8+ T lymphocytes, which eliminate infected and tumor cells (Fraser et al., 2004). The humoral immune response occurs when B lymphocytes, which reside and circulate in the lymph nodes and spleen, upon encountering their specific antigen, bind through their receptor and induce the activation of the B lymphocyte, producing the activation and subsequent production of antibodies, as well as the internalization of the antigen and subsequent antigenic presentation. The B lymphocyte then becomes an APC that presents the antigen to its specific T helper lymphocyte, which secretes cytokines (Fraser et al., 2004; Maloney et al., 2020).

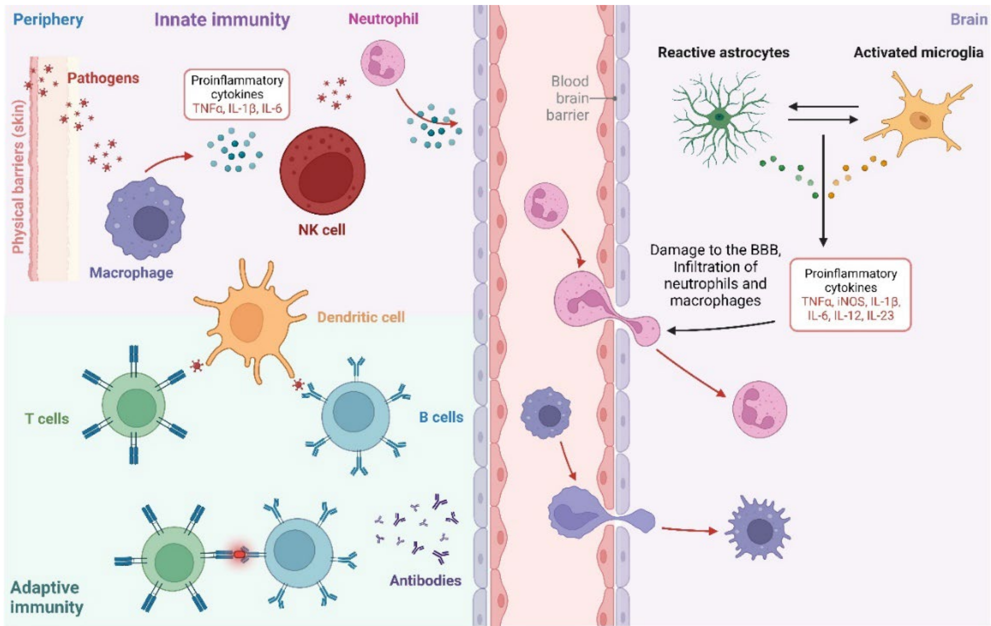

Figure 5. Components of the innate and adaptive immune system. The innate immune system will be activated if pathogens cross physical barriers. Innate immune cells are NK and phagocytic cells (macrophages and neutrophils) that have receptors that recognize pathogens and damaged or infected cells. Once activated, they initiate processes aimed at destroying the pathogen, such as phagocytosis, in addition to releasing cytokines and chemokines that will attract cells and fluids to the affected site, causing inflammation. Finally, cytokines can enter the brain through the blood-brain barrier, activating microglia. Activated proinflammatory microglia also release astrocyte-activating signals that induce neuroinflammatory astrocytes that, in turn, amplify the neuroinflammatory cycle. When pathogens cannot be neutralized, dendritic cells can stimulate cells of the adaptive immune system by presenting them as antigens. When activated, T and B cells secrete antibodies that neutralize the extracellular microorganisms. The generation of cellular memory in T and B cells results in a specific and rapid immune response in case of recurrent infection.

From an immunological point of view, the CNS has special characteristics. The CNS possesses the BBB, which in the absence of an inflammatory process prevents the entry of immune system cells into the CNS (Engelhardt et al., 2017). The BBB is a structural and functional barrier that exists between blood and the nervous tissue of the CNS. The key element of the BBB is the endothelial cells, which at this site are intimately bound by tight junctions. Capillaries thus play an essential role in the

selective restriction of exchanges between blood and the CNS. In addition, astrocyte extensions play a secondary role in the formation of the BBB. In addition to the BBB, certain factors cause the brain to remain immuno-inhibited: the absence of the lymphatic system, the low expression of molecules capable of presenting antigens to T lymphocytes and the immunosuppressive cytokines secreted by astrocytes lining and contacting the BBB (Griffiths et al., 2010; Shechter et al., 2013). In this system, the first line of immune defense is constituted by microglia, belonging to the manocyte-macrophage system. Microglia cells are the only cells of the immune system present in the CNS in a healthy state. They are a type of macrophage with phagocytic function capable of being activated in different ways. Microglia remove debris from damaged tissue and can perform tissue repair and secrete neurotrophic and protective factors (BDNF). Microglia are activated by immune system cells that, after an inflammatory process, can cross the BBB. When activated, they increase their phagocytic activity and trigger the production of cytokines that complete the inflammatory cascade (Andreasson et al., 2016).

When this complex defense system fails, the so-called immune disorders appear, which are a group of diseases in which the immune system mechanisms are altered or absent (Ilchmann-Diounou & Menard, 2020).

5.2. Activation of the immune system due to social stress

Selye, in 1936, demonstrated that after exposure of the organism to adverse conditions for prolonged periods, what he called the stress triad occurs: adrenal hypertrophy, gastric ulceration and hypertrophy of the thymus and lymph nodes. It was later observed that during the adaptation and exhaustion phases of the stress syndrome there is an inhibition of the immune system as part of the normal response of the organism to aversive stimulation (Selye, 1936). Glucocorticoids and the neurohormones epinephrine and NE, in an attempt to restore the organism's homeostasis and cope with the stressful situation, inhibit the functioning of systems

with higher energy expenditure, such as the digestive, growth and immune systems (Padgett & Glaser, 2003). Thus, during the exposure of the organism to stress, there is a hypofunction of the immune system, leaving the organism exposed to the action of infectious agents in the environment, and more susceptible to disease (Rohleder, 2014). The main mediators of the immunomodulatory effects of stress are glucocorticoids and the catecholamines, epinephrine and NE, which exert a direct influence on the functioning of immune cells by coupling to their specific receptors, located in the cytoplasm and cell membrane respectively. They also exert indirect effects by altering the production of cytokines such as INFy, tumor necrosis factor (TNF) and interleukin (IL)-1, IL-2 and IL-6, all of which are necessary for the maturation and mobilization of lymphocytes and other immune cells (Sorrells et al., 2009). Primary and secondary lymphoid organs, as well as T and B lymphocytes, neutrophils, monocytes and macrophages, possess type II glucocorticoid receptors for corticosteroid hormones (Pace & Miller, 2009). Glucocorticoids, upon coupling to their cytoplasmic receptors on immune system cells, translocate to the nucleus and function as transcription factors for numerous proteins synthesized by lymphocytes, macrophages and other immune system cell types. Among the proteins whose genes possess glucocorticoid response elements are cytokines and immune cell surface receptors and antigens (Pace & Miller, 2009).

Currently, the term neuroinflammation is used to refer to a series of events that produce molecular and cellular modifications in the form of an immune response in the CNS. The process of neuroinflammation has gained great relevance in recent decades as it has been linked to the development of mental and neurodegenerative diseases. In the 1990s, the so-called neuroinflammatory theory of depression was outlined (see, for example, Maes et al., 2009), which is based on the increased inflammatory mediators observed in patients with depression and the occurrence of depression or anxiety in subjects who have been injected with cytokines. Interest in the role of the immune system as a mediator of the negative effects of social stress

has been increasing in recent years, and there are now numerous studies demonstrating its role as a mechanism by which increased vulnerability to mental illnesses such as mood disorders and addictions occurs (Cathomas et al., 2019). In animal models, psychological stressors, such as SD, restraint and chronic unpredictable stress, alter the peripheral immune responses. These studies have observed increases in monocyte, neutrophil, IL1-β, IL-6, IL-13, TNFα and IL-10 levels, and decreases in dendritic cells and promotion of T-lymphocyte apoptosis (Ambreé et al., 2018; Finnell et al., 2017; Heidt et al., 2014; Pfau et al., 2019; Tsyglakova et al., 2019). In particular, some of these immune responses are specific for animals susceptible to social stress-induced depressive-like behaviors (Ambreé et al., 2018, Ballestín et al., 2021). Similar effects have been observed in the CNS, where a wide variety of psychosocial stressors increase IL1-β and IL-6 expression in various brain regions, activate microglia, and influence mast cell activation throughout the brain (Ballestín et al, 2021; Bollinger et al., 2017; Hellwig et al., 2016; Rodriguez-Arias 2017; Wohleb et al., 2012). Chronic stress can also alter the integrity of the BBB, increasing the entry of peripherally derived monocytes into the brain, as well as altering the stress responsiveness of immune cells, modulating the expression of their glucocorticoid receptor and reducing the expression of tight junction protein claudin-5 and increased basal laminin degradation in the NAc and hippocampus (Ataka et al., 2013; Jung et al., 2015; Rodriguez-Arias 2017). Lehmann et al. (2020) observed microhemorrhages in the BBB in mice susceptible to the effects of SD. In contrast, no BBB alterations were observed in resilient mice, indicating certain vascular adaptations that maintain BBB integrity (Lehmann et al. 2020).

Microglia, astrocytes and mast cells are particularly sensitive to GCs and express GRs and mineralocorticoid receptors (Sierra et al., 2008; Stein et al., 2017). GCs stimulate microglia proliferation, up-regulate activation and inflammatory markers such as MHCII, CD14, CD86 and TLR4, acting through NMDA, β-adrenergic and

IL-1β receptors (de Pablos et al., 2006; Frank et al., 2012; Nair & Bonneau, 2006; Wohleb et al., 2012). The release of these proinflammatory markers in turn lead to astrogliosis, causing structural and functional changes in the brain (Calcia et al., 2016). In mice exposed to SD, long-term changes in microglia activation have been observed 3 weeks after the last SD. In both NAc and PrL, a more pronounced activation of microglia was observed, with a decrease in M1 and M2 morphotypes and an increase in M3-5 in defeated mice (Rodriguez-Arias et al. 2018). Social stress also induces alterations in astrocytes. GCs alter the quantity and gene expression of astrocytes in the brain (Carter et al., 2012; MacDonald et al., 2019; Piechota et al., 2017). Repeated SD also induced a long-lasting decrease of glial fibrillary acidic protein (GFAP)+ cells in the NAc, while an increase of synaptophysin was observed in the PrL (Rodriguez-Arias et al. 2018).

Given the importance of the immune response in normal behavior and neuronal function, a mismatch in the functioning of the immune system induced by prolonged social stress over time is key in the development of pathological disorders such as depression, anxiety or SUD (Reddaway & Brydges, 2020). Primarily, short-term social stress-induced changes in the immune response have been studied, but some studies show that these changes may be long-lasting and lead to permanent changes in the developing immune system (Lo Iacono & Carola, 2018).

5.3. Vulnerability to addiction induced by the neuroinflammatory response to social stress

As discussed in previous sections, social stress influences the vulnerability to develop a SUD and modifies the response to rewarding drug stimuli. In the last decade, structural and functional changes in the brain, associated with the release of proinflammatory cytokines and chemokines leading to microglial activation and astrogliosis (Calcia et al., 2016), have been linked to vulnerability to the development of an addictive disorder. The immune response in the CNS affects

dopaminergic signaling in the mesolimbic pathway, modifying reward behaviors. It has been observed in depressed patients that plasma levels of C-reactive protein and inflammatory cytokines correlated negatively with resting-state connective functionality between the NAc and PFC, reward sensitivity, and psychomotor function (Felger et al., 2016; Goldsmith et al., 2016). For example, in patients chronically treated with IFN- α, half were observed to meet criteria for the diagnosis of depression. They showed decreased ventral striatum activity to reward, were less sensitive to reward, and showed fatigue and motor slowing (Capuron et al., 2012). In rodents, it has been reported that after an inflammatory process, there is a reduction in sucrose preference, consumption and motivation, as well as a reduction in DA synthesis and release (Kitagami et al., 2003; Reinert et al., 2014). Following chronic SD exposure, increases in microglia activation in the VTA and decreased DA turnover in the NAc have been observed (Tanaka et al., 2012). There are strong data showing that, after repeated SD exposure in mice, there is an increase in the neuroinflammatory response through increases in IL-6 levels and decreases in CX3CL1 in striatum, PFC and hippocampus, which induce an increase in vulnerability to the rewarding response to cocaine (Ballestín et al., 2021; Ferrer-Pérez et al., 2018a, 2019a, 2019b; Giménez-Gómez et al., 2021). Interestingly, this effect has not been observed in mice resilient to repeated SD-induced depressive-like behaviors (Ballestín et al., 2021; Giménez-Gómez et al., 2021). Although less studied, modulation of the immune system in alcohol consumption following exposure to social stress has also been demonstrated. For example, double deletion of IL-1RI and TNF-1 receptors blocked the increase in alcohol consumption induced by chronic SD, highlighting the contribution of these receptors in the regulation of alcohol intake by the negative reinforcement of social stress (Karlsson et al., 2017).

Another component of the neuroinflammatory response studied in relation to substance abuse and SD exposure is the TLR4 receptors of the innate immune system, which recognize pathogen-associated molecular patterns. SD exposure

activates these receptors that modulate transcription factors linked to neuronal plasticity, memory and neurotoxicity (Nie et al., 2018). For example, an increase in the transcription factor NFk, which is involved in various stages of the addictive process, has been observed in the NAc, striatum and hippocampus of mice subjected to repeated SD (Montagud-Romero et al., 2021). KO mice lacking the TLR4 receptor do not show increases in cocaine or alcohol consumption that induce SD (Blednov et al., 2011; Montagud-Romero et al., 2021). Finally, we must remember the neuroinflammatory potential of alcohol. Alcohol is able to activate the inflammatory response mainly through recruitment of TLR4 from glial cells, astroglia and microglia to induce endocytosis, causing receptor internalization and trafficking. Activation of these receptors by alcohol promotes rapid activation of MAPK and NF-κB signaling, which induces the generation of cytokines (e.g., pro-IL-1β, IL-6, IL-8, and TNF-α), chemokines (e.g., MCP-1), and inflammatory mediators (iNOS and COX-2). In addition, alcohol activates the NLRP3 inflammasome in glial cells, inducing the release of pro-IL-1β through TLR4, amplifying the neuroinflammatory response (for a review, see Pascual et al., 2021). In contrast, in studies in TLR4-KO mice, no neuroinflammatory effects induced by ethanol consumption have been observed (Montesinos et al., 2016, 2015; Pascual et al., 2015). Thus, the results obtained to date point to the immune system as an important therapeutic target for treating addiction-related problems resulting from exposure to social stress.

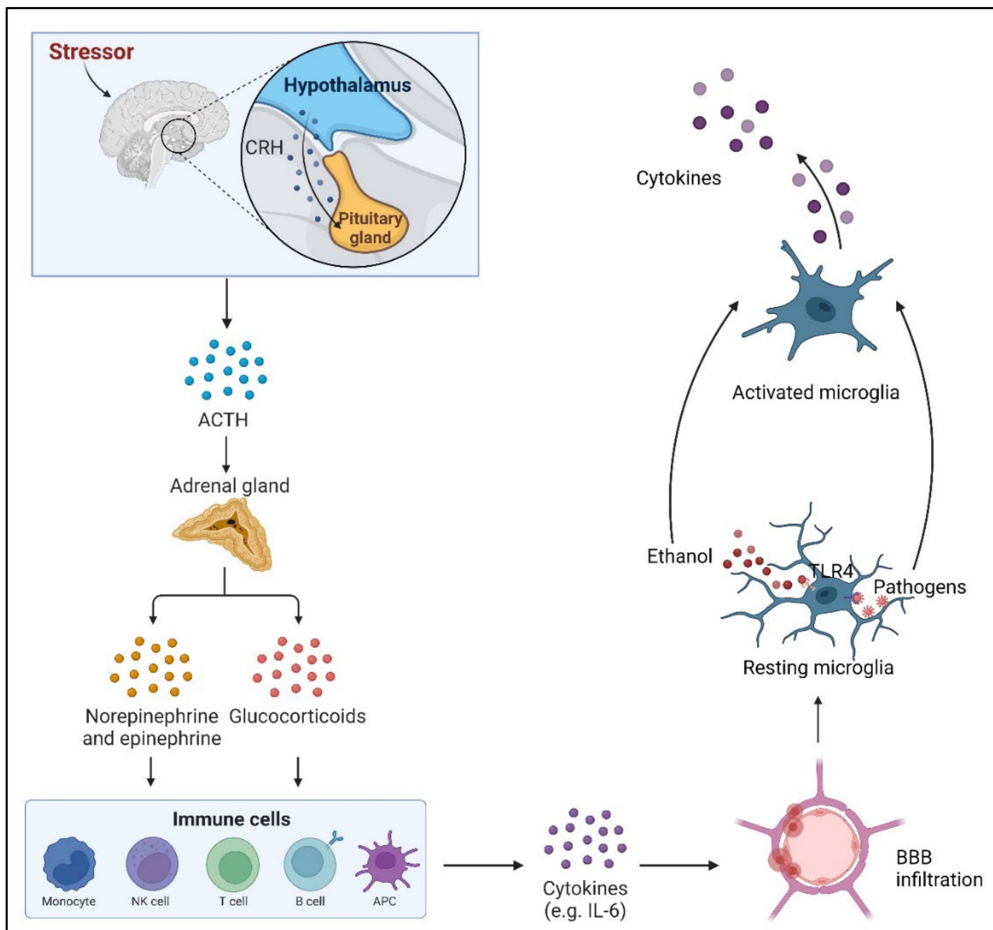

Figure 6. Vulnerability to addiction induced by the neuroinflammatory response to social defeat. Stress activates the HPA axis, which induces the release of glucocorticoids, norepinephrine and epinephrine. Glucocorticoids and catecholamines act as immunomodulatory mediators of stress, influencing immune cell function and increasing the release of proinflammatory cytokines and chemokines that can cause BBB damage. This damage allows cytokine infiltration and activation of microglia. Alcohol consumption promotes microglial activation and cytokine release through its interaction with TLR.

It should be noted at the end of this section that, contrary to what occurs in animals susceptible to depressive-like behaviors, resilient mice to social stress show a less pronounced neuroinflammatory response after exposure to acute or chronic stress. Numerous studies have revealed that resilient mice exhibit decreased protein levels

of proinflammatory cytokines, such as IL-6, and increased levels of chemokines, such as CX3CL1 (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Hodes et al., 2014), as well as increased anti-inflammatory cytokines, such as IL-7 and IL-10 (Stewart et al., 2015). Furthermore, some of these studies have corroborated that these resilient animals show a dampened neuroinflammatory response and do not develop vulnerability to addictive behaviors towards cocaine (Ballestín et al., 2021; Giménez-Gómez et al., 2021).

In summary, resilience to the effects of SD seems to depend, in part, on the neuroinflammatory response and is characterized by a less reactive phenotype. Furthermore, these results corroborate the importance of the immune system as a mechanism by which increased vulnerability to substance abuse induced by social stress may occur.

6. Strategies for promoting resilience to the effects of social defeat

6. Strategies for promoting resilience to the effects of social defeat

Promoting and enhancing adaptive coping mechanisms in stressful situations is a powerful modulator of mental health. Enhancing resilience management can be improved through environmental and pharmacological interventions, or a combination of both. In support of and in combination with psychological intervention by mental health professionals, numerous preclinical research teams have studied the mechanisms underlying the behavioral and neural effects induced by social stress and pharmacological and environmental interventions that can help to improve resilience in the most vulnerable individuals. These interventions can be performed at any stage of the life cycle, and researchers can focus on critical periods of increased vulnerability such as adolescence. As mentioned above, the neuroinflammatory theory of depression has gained great relevance in recent decades, as it has been related to the development of mental illnesses (such as depression, anxiety, and addiction) and neurodegenerative diseases, and there are currently numerous studies that demonstrate its role in the effects induced by social stress (Maes et al., 2009). Because of this relationship between neuroinflammation, stress and substance abuse, some researchers have employed non-steroidal anti-inflammatory drugs, such as indomethacin, to reverse the increased conditioned rewarding effects of cocaine induced by social stress (Ferrer-Pérez et al., 2018a; Giménez-Gómez et al., 2021).

Another pharmacological intervention that appears to be a really promising therapeutic target in both humans and rodents is the administration of the hormone oxytocin (OXT) to reduce the negative effects of social stress in coping with adverse situations and in the treatment of addictive disorders. Signals that promote CRH synthesis in the PVN also promote OXT synthesis and are driven to the anterior pituitary, much like CRH (Bao et al., 2008; Leistner & Menke, 2020). The release of OXT from the PVN has the ability to inhibit or dampen, i.e., modulate the HPA

axis response (Gobrogge & Wang, 2015; Heck et al., 2020; Wang et al., 2019; Winter & Jurek, 2019). OXT exerts its role in the adaptive response to social stress by decreasing the stress response through regulation of CRH. We can conclude that social stress modifies the projection of OXT neurons and alters plasma levels of OXT in both humans and animals. These changes result in the dysfunction of OXT's primary role in stress, which is the modulation of the HPA response and reduction of the impact of the stress response. Furthermore, clinical and preclinical evidence suggests that repeated drug exposure leads to a decrease in OXT levels that appears to be related to a decrease in its synthesis (Lee et al., 2017). Finally, OXT has also been reported to inhibit proinflammatory mediators such as TNF-α, IL-1β and nitric oxide synthase (Akman et al., 2015; Amini-Khoei et al., 2017; Ferrer-Pérez et al., 2019a; Karelina et al., 2011; Yuan et al., 2016; Wang et al., 2018).

Promising resilience-enhancing environmental interventions that increase stimulation and provide richer and more varied opportunities for interaction with the social and physical environment have also been studied in the recent decades and have been shown to exert a variety of long-term effects at the neuroanatomical, neurochemical, and behavioral levels in several animal species. Moderate and controlled physical exercise can be a great preventive strategy to control stress levels and stress-induced substance use. WHO (2020) emphasizes the importance of physical exercise at all stages of the life cycle, showing that, in adolescence, physical activity improves cognitive outcomes (academic performance and executive function) and mental health (reduction of depressive symptoms). In preclinical studies, voluntary exercise on running wheels has been shown to have an enhancing effect on learning and neurogenesis, resulting in increased neurotrophic factors and changes in molecular signaling, as well as a reduction in depressive-like behaviors (Salam et al., 2009; Mul, 2018; Lynch et al., 2019; Pietrelli et al., 2018; Zolfaghari et al., 2021). Other studies focused on the reward system have shown that physical

exercise modulates gene transcription in dopaminergic neurotransmission in the mesolimbic pathway (Greenwood et al., 2011), and reduces the consumption of substances of abuse such as alcohol (Darlington et al., 2014, 2016; Ehringer et al., 2009; Gallego et al., 2015). In addition, physical exercise also regulates the immune response by up-regulating BBB tight junction-associated proteins and protects the brain from injury by reducing microglia activation and cytokine levels in the hippocampus in mice (Park et al., 2016; Spielman et al., 2017) and humans (Paolucci et al., 2018) and has the ability to decrease inflammatory markers and oxidative stress by reducing TLR expression in monocytes and macrophages, with a consequent decrease in the reactivity of these immune cells (Eyre & Baune, 2012; Gleeson et al., 2011). In addition to the anti-inflammatory potential, physical exercise modulates monoamine metabolism and decreases basal levels of GC (Eyre & Baune, 2012).

Environmental enrichment (EE) refers to improved housing conditions for animals through the use of larger cages containing objects and different spaces that facilitate play and exploration, while allowing the animals greater control over their environment. EE facilitates the sensory, cognitive and motor stimulation of animals relative to standard housing conditions. It has been identified as a protective factor in the prevention and treatment of some stress-related emotional disorders, such as depression, and may mediate the inflammatory response. Also, in general terms, it is an experimental model that has been shown to have protective effects against the consumption and rewarding effect of drugs, as well as to reduce the behavioral effects produced by drugs of abuse. The beneficial effects of EE that could explain the protective effect on increased alcohol intake and the neuroinflammatory response include increased neurogenesis with elevated expression of BDNF (Novkovic et al., 2015; Schloesser et al., 2010) and increased synaptic and transcriptomic capacity (Hüttenrauch et al., 2016; Zhang et al., 2018). Exposure to EE during adolescence

could also change the dynamics of social interaction, sensory processing, and mechanisms underlying early stress, with a decrease in CRHR1 genes and an increase in hippocampal CRHR2 observed in male rats housed in EE conditions (Kentner et al., 2018). Among other factors, the facilitation of problem-solving ability and immunoreactive responsiveness to EE-induced OXT in male rats should also be considered (Neal et al., 2018). On the other hand, attenuations of IL-6 and IL-β1 increase in prefrontal mRNA expression induced by moderate social stress have been observed in animals under EE conditions (McQuaid et al., 2018).

Finally, the negative consequences of early life stress are well recognized in contemporary perspectives on human mental health. Chronic exposure to severe forms of stress has been linked to the development of mood disorders, anger, anxiety, and substance abuse (Anda et al., 2006; Heim et al., 2008; Turner & Lloyd, 2003). Much less researched, but of equal importance, is the evidence that early and intermittent exposure to stress does not generate vulnerability, but rather enhances arousal regulation and resilience. Variously described in human studies as inoculation (Dienstbier, 1989; Masten, 2001; Mortimer & Staff, 2004; Rutter, 2006), the notion that intermittent exposure to stress in early life induces resilience has its roots in the observations of Levine et al., first described in the mid-1950s (Levine, 1957; Levine et al., 1967). Despite its negative connotations, stress has an adaptive value, as it promotes homeostasis. Exposure to stressful events that are not devastating, but are challenging enough to elicit emotional instigation and cognitive processing, can promote successful coping with subsequent stressors. In the scientific literature, this phenomenon has been termed "stress inoculation" (Lyons et al., 2010; Meichenbaum, 2017) and can be applied across several animal models, such as brief maternal separation, exposure to predator odor, and exposure to sensory components of SD during adolescence and adulthood. Although this hypothesis has great potential and is applicable at any stage of the life cycle, it remains relatively understudied at present.

2. AIMS AND HYPOTHESES

2. Aims and hypotheses

As we have observed in the previous sections, social stress is the main risk factor for addictive behaviors. Stress-susceptible phenotypes have become in the last decade a target of study for the application of effective treatments to enhance stress resilience. Promoting active coping through positive pharmacological and environmental interventions is scientifically validated in the treatment of addictions. However, these interventions to promote resilience during adolescence as a preventive measure for intense stressful experiences during adulthood have not been widely studied.

The main aim of the present Doctoral Thesis was ***to develop pharmacologic and environmental strategies to enhance resilience to the negative effects induced by social defeat on behavior and the neuroinflammatory response.***

To reach this aim, we first confirmed and characterized the neuroinflammatory potential of social defeat stress and its mediation of increased ethanol consumption using the operant paradigm of oral self-administration. Subsequently, based on previous reports from our laboratory and others, we assessed the potential of oxytocin administration or physical exercise to buffer the negative effects of social defeat on ethanol consumption and the neuroinflammatory response. After confirming that these interventions potentiate resilience to the deleterious effects of social defeat, we set out to characterize the preventive potential of exercise, environmental enrichment or stress inoculation during adolescence. These studies further characterized the vulnerability and resilience to social stress experience during adolescence in mice, since the response to social defeat is complex and unique during this stage of life. Finally, a new line of research was initiated during an international research stay to further characterize resilient/susceptible phenotypes, focusing on the study of neuronal and synaptic morphological changes and astrogliosis in key brain structures.

Identifying the subjects most vulnerable to the effects of social stress, as well as determining the potential of interventions primarily delivered during adolescence and prior to stressful life experiences in adulthood, will contribute to the development of individualized preventive and therapeutic therapies in the treatment of addictive disorders.

Following is a description of the principal aims and hypothesis of the seven studies that compose this doctoral thesis.

Study 1. The aim of the first study was to evaluate whether pretreatment prior to social defeat with the neuropeptide oxytocin could protect against ethanol intake and the neuroinflammatory response induced by social defeat. To that end, we administered 1 mg/kg of oxytocin half an hour before each social defeat.

Hypotheses:

- Defeated mice will show increased CX3CL1 and CXCL12 protein levels in the striatum after the fourth social defeat.
- Social defeat will induce long-term increased consumption and motivation to obtain ethanol in operant oral self-administration of ethanol (6%).
- Defeated mice will show increased CX3CL1 and CXCL12 protein levels in the striatum after termination of oral ethanol self-administration.
- Oxytocin will prevent the increase in CX3CL1 and CXCL12 protein levels in the striatum after the first and fourth social defeats.
- Oxytocin will prevent the increase in ethanol consumption and motivation to obtain the substance induced by social defeat.
- Oxytocin will prevent the increase in CX3CL1 and CXCL12 protein levels in the striatum after oral ethanol self-administration.

Study 2. The aim of the second study was to assess whether exposure to voluntary and controlled physical activity is an effective intervention to prevent and reduce the long-term consequences induced by social defeat on ethanol consumption and on the neuroinflammatory response of the CX3CL1 and CXCL12 chemokines. To this end, half of the sample was subjected to physical exercise by accessing a low-profile running wheel 1h per day 3 times per week during the whole experimental procedure.

Hypotheses:

- Defeated mice will show increased CX3CL1 and CXCL12 protein levels in the striatum after the fourth social defeat.
- Social defeat will induce long-term increased consumption and motivation to obtain ethanol in operant oral self-administration of ethanol (6%).
- Defeated mice will show increased CX3CL1 and CXCL12 protein levels in the striatum after termination of oral ethanol self-administration.
- Passive coping during social defeat sessions will correlate with increased ethanol consumption during the FR1 program of oral ethanol self-administration.
- Access to the voluntary running wheel will counteract the increase in ethanol consumption and the motivation to obtain the substance induced by social defeat.
- Access to voluntary running wheels will prevent social defeat-induced increase in chemokine levels in the striatum.

Study 3. The objective of the third study was to assess the effect of environmental enrichment during adolescence on the behavioral and neuroinflammatory consequences of social defeat. To do that, we first evaluated and characterized the susceptible/resilient phenotypes to behavioral and neuroinflammatory effects induced by social defeat. To classify the phenotypes, social withdrawal was assessed in the social interaction test 24 h after the last social defeat.

Hypotheses:

- Mice subjected to social defeat will show a resilient or susceptible phenotype depending on their avoidance or social interaction behaviors in the social interaction test.
- Mice susceptible to depressive-like behaviors will show an increased response in operant ethanol self-administration.
- Social defeat will produce an increased neuroinflammatory response in susceptible mice.
- Environmental enrichment will counteract the effects induced by social stress in susceptible mice.
- Environmental enrichment will counteract the increased ethanol intake on oral self-administration in susceptible mice.
- Environmental enrichment will decrease the neuroinflammatory response of susceptible mice.

Study 4. The aim of the fourth study was to assess the preventive effect of physical exercise to promote resilience to the increase of ethanol consumption and the changes of BDNF induced by social defeat. For this purpose, mice were subjected to physical exercise 1h per day three times per week from adolescence until the first social defeat in adulthood.

Hypotheses:

- Mice susceptible to depressive-like behaviors without access to physical exercise will show increased ethanol consumption and decreased BDNF protein levels in the striatum and hippocampus.
- Access to physical exercise will promote an increase in the percentage of mice resilient to social defeat-induced depressive-like behaviors.
- Access to physical exercise will decrease ethanol consumption in the D*rinking in the Dark* paradigm in susceptible mice.
- Access to physical exercise will decrease ethanol consumption and motivation for ethanol in oral self-administration in susceptible mice.
- Access to physical exercise will restore BDNF protein levels in the striatum and hippocampus.

Study 5. The aim of the fifth study was to assess whether inoculation with mild social stress during adolescence can increase resistance to other stressors of higher intensity in adulthood. For this purpose, adolescent mice were exposed to a single social defeat in adolescence. Subsequently, in adulthood, mice were subjected to the social defeat protocol. Three weeks later, oral ethanol self-administration was assessed and the inflammatory response of IL-6 and CX3CL1 was analyzed.

Hypotheses:

- Stress inoculation in adolescence will increase the percentage of mice resilient to depressive-like behaviors after exposure to social defeat in adulthood.
- Mice susceptible to depressive-like behaviors exposed to stress inoculation will reduce ethanol consumption in the *Drinking in the Dark* paradigm.
- Mice susceptible to depressive-like behaviors subjected to stress inoculation will reduce ethanol consumption and motivation on oral self-administration induced by social defeat.
- Mice susceptible to depressive-like behaviors subjected to stress inoculation will show a decreased neuroinflammatory response induced by social defeat.

Study 6. The aim of the sixth study was to characterize the resilient/susceptible phenotype in mice exposed to social defeat during adolescence. A social defeat procedure similar to that employed in the previous studies was used, but the encounters with the intruder mice took place from postnatal day 27 to 36. Three weeks later, one set of mice was subjected to conditioning of place preference with 1.5 mg/kg cocaine and another set to oral self-administration of ethanol (6%). At the end of both paradigms, brains were removed and IL-6 and CX3CL1 levels in prefrontal cortex and striatum were analyzed to assess the neuroinflammatory response induced by social defeat.

Hypotheses:

- Adolescent mice will show a resilient or susceptible phenotype to social defeat-induced depressive-like behaviors in the social interaction test.
- Adolescent susceptible mice will show an elevated anxiogenic response compared to resilient mice.
- Adolescent mice susceptible to depressive-like behavior will show an increased response to cocaine reward.
- Adolescent mice susceptible to depressive-like behavior will show increased ethanol consumption.
- In adolescent susceptible mice, an increased neuroinflammatory response will be observed.

Study 7 (IBCN, UBA). The aim of the seventh study was to analyze the neuronal and synaptic morphological characteristics and astroglial response of individuals susceptible/resilient to the negative effects of social defeat on the reinforcing actions of ethanol. Therefore, the aim was to explore whether there are morphological differences between susceptible and resilient mice. To carry out this study, brains of male mice subjected to social defeat, classified as resilient/susceptible according to their social interaction test scores, and evaluated in the paradigm of oral ethanol self-administration (20%), were sent to the Institute of Cell Biology and Neuroscience (IBCN, Buenos Aires, Argentina). Once there, both the synaptic and neuronal morphology and astroglial response of the animals were analyzed by immunofluorescence of several markers (MAP-2, NF200, GFAP and S-100β) in the prelimbic cortex, striatum and Cornu Ammonis 1 (CA1) of the hippocampus.

Hypotheses:

- Mice susceptible to social-defeat-induced depressive-like behaviors will consume greater amounts of ethanol.
- Mice susceptible to social-defeat-induced depressive-like behaviors will exhibit decreased dendritic arborization, showing decreased MAP-2 immunostaining.
- Mice susceptible to social-defeat-induced depressive-like behaviors will show altered axonal cytoskeleton, showing reduced neurofilament NF200 immunostaining.
- Mice susceptible to social-defeat-induced depressive-like behaviors will show increased astrogliosis, showing increased immunostaining of GFAP and S-100β proteins.

3. MATERIAL AND METHODS

3. Material and Methods

3.1. Subjects

In this doctoral thesis, mice of the C57BL/6J and OF1 strain were used (Charles River, France). The total number of mice is specified in each of the studies presented in the Results section. The experimental mice were delivered to our laboratory at PND 21 (in the studies 2, 3, 4, 5, 6 and 7) and at PND 42 (Study 1). All mice (except those used as aggressive opponents) were housed in groups of four or five in plastic cages (25 × 25 × 14.5 cm), except in Study 3 where during PND 21 to 47 they were housed in environmental enrichment cages (27 × 27 × 14 cm). In studies 1, 2 and 7, OF1 strain mice were used as experimental subjects and in studies 3, 4, 5 and 6, C57BL/6J strain mice were used. OF1 mice used as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998). All mice were housed under the following conditions: constant temperature and humidity, a reversed light schedule (lights off at 08:00 and on at 20:00), and food and water were freely available *ad libitum*, except during the behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committee (University of Valencia).

Figure 7. Strains of mice used. On the left, strain OF1; on the right, train C57BL/6J.

3.2. Drugs

In studies 1, 2, 3 and 4, during the SA training phase, a 0.2% (w/v) saccharin (Sigma-Aldrich, Madrid, Spain) solution in water was used. During the SA substitution phases, a mixture of 0.15% saccharin concentration dissolved in water and 2% ethanol was used for the first subphase; in the second subphase, a mixture of 0.10% saccharin solution in water and 4% ethanol was used; and, in the third subphase, a mixture of 0.05% saccharin solution in water and 6% ethanol was used. During fixed ratio 1 and 3, and progressive ratio schedules of SA, absolute ethanol (Merck, Madrid, Spain) was diluted in water using 6% ethanol solution.

In studies 5, 6 and 7, absolute ethanol (Sigma-Aldrich, Madrid, Spain) was diluted in water using 20% ethanol solution for the Drinking in the dark procedure and SA.

Moreover, in study 1, mice were injected with 1 mg/kg of OXT (Sigma-Aldrich, Madrid, Spain) in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs.

3.3. Social Defeat

The mice in the stress/defeat groups were exposed to four SD episodes. The social defeat sessions were performed in all studies at PND 47, 50, 53 and 56, with the exception of study 6, in which it was performed during PNDs 27, 30, 33 and 36. The initial phase began by introducing the “intruder” (the experimental animal) into the home cage of the “resident” (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-min period. Finally, the wire mesh was returned to the cage to separate the two animals

once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al., 1998).

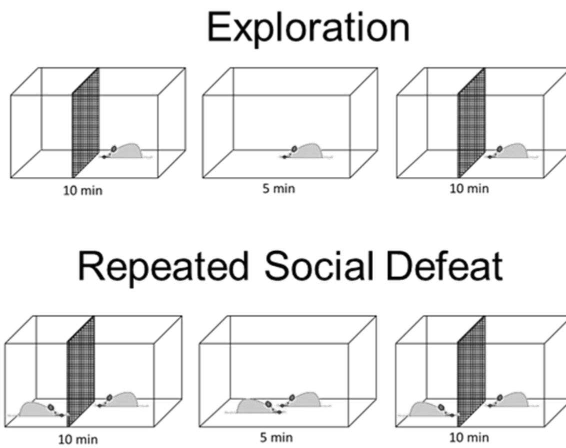

Figure 8. Repeated social defeat procedure. Four sessions every 72h. It consists of 3 phases: 10 min of sensory interaction; 5 min of agonistic interaction; 10 min of sensory interaction. The control group is exposed to the same procedure without resident mouse.

3.4. Elevated Plus Maze

The elevated plus maze (EPM) test was carried out essentially following the procedure described by Daza-Losada et al. (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, mice were habituated to the experimental room for 1 h prior to testing. At the beginning of each trial, the experimental mice were placed on the central platform facing an open arm and were allowed to explore for 5 min. The behavior displayed by the mice during the test was recorded by an

automated tracking system (EthoVision XT 11, Noldus) that tracks the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms were measured to characterize the anxiolytic effects of the SD (Ferrer-Pérez et al., 2018b; Rodríguez-Arias et al., 2016).

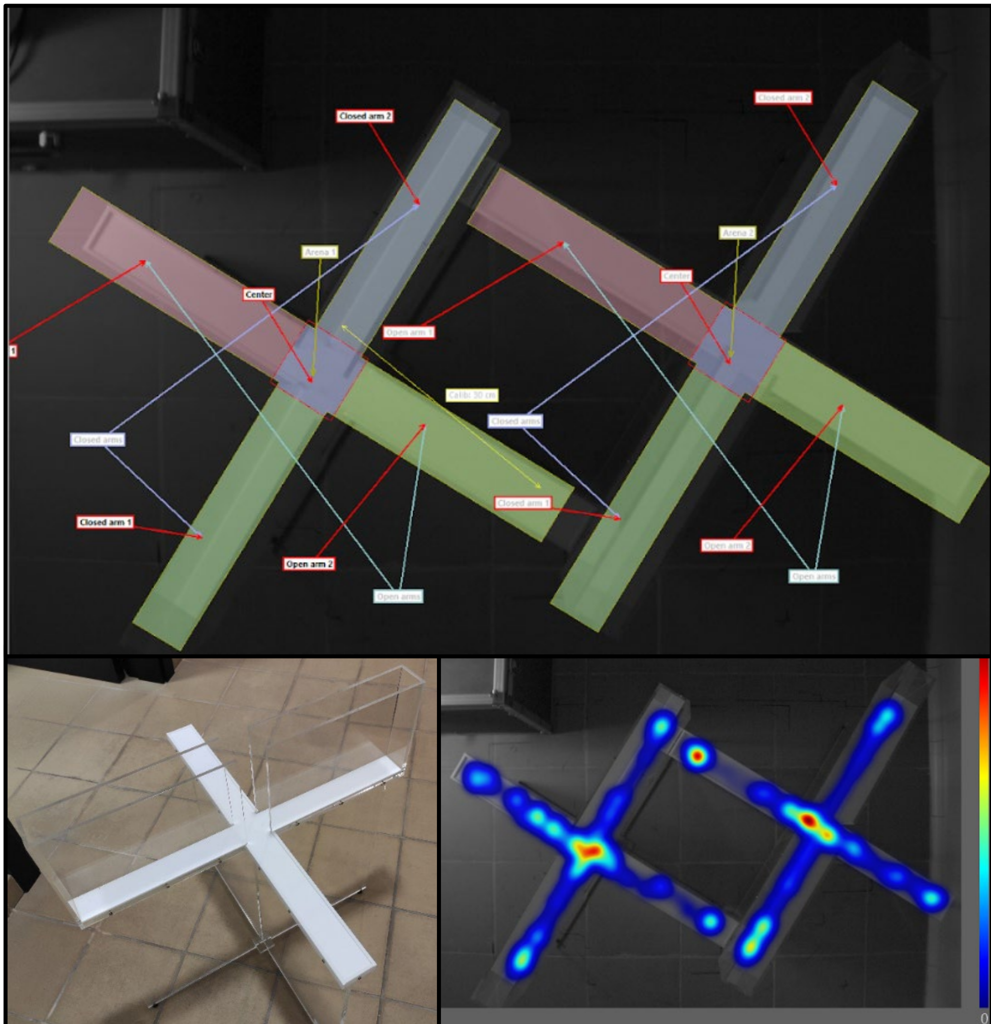

Figure 9. Elevated plus maze. Above, Ethovision template. Bottom left, photograph of the maze. Below right, heatmaps generated using EthoVision.

3.5. Social Interaction Test

The social withdrawal ratio used was based on the social approach-avoidance test previously described by Berton et al., 2006. The test took place 24 h after the last SD during dark cycle and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, Noldus, 50 fps; camera placed above the arena). Animals were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10×6.5×35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Before each session, the arena was cleaned with 5 % alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

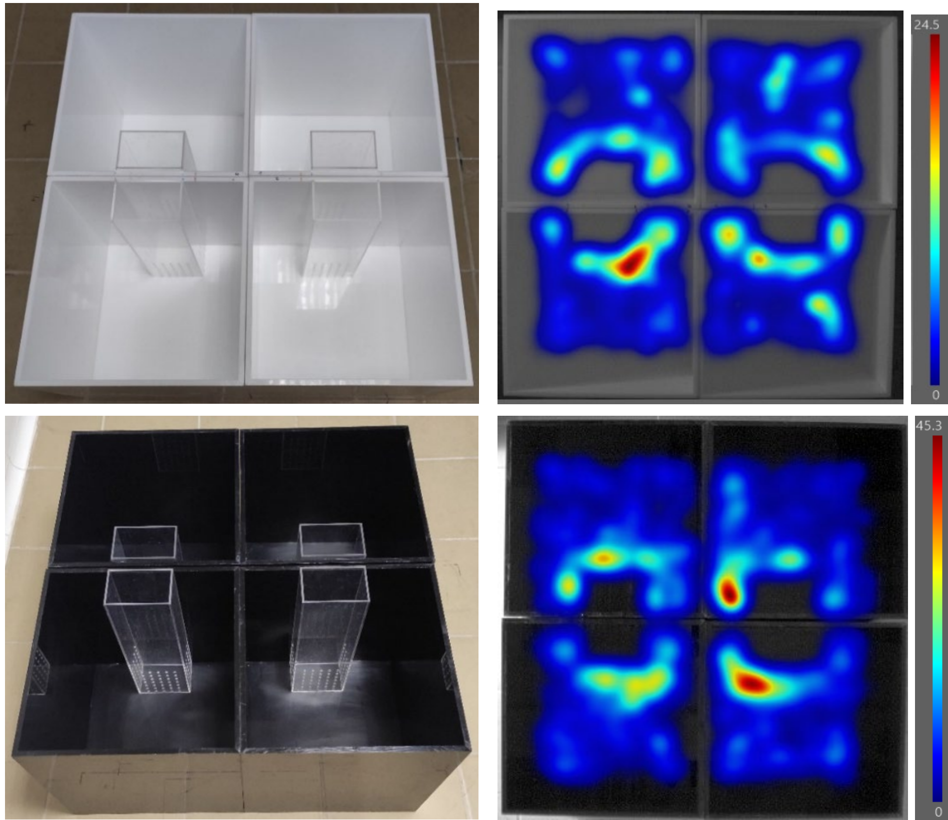

Figure 10. Social Interaction Test. On the left, images of the test cages. On the right, heatsmaps generated using EthoVision.

3.6. Pharmacological and environmental interventions

3.6.1. Oxytocin administration

In study 1, a pharmacological intervention administering the neuropeptide OXT was applied to evaluate its effect on the neuroinflammatory response induced by SD after the first and fourth defeat and after the end of oral ethanol SA. To this end, mice received an i.p. injection of saline or 1 mg/kg of OXT 30 min before each social defeat or exploration session.

3.6.2. Voluntary wheel running

The type of wheel used was the low-profile running wheel (Med Associates Inc.), which rotates on a central axis in a horizontal plane, allowing physical activity to be carried out through natural exercise as in spontaneous locomotion. These wheels have an ideal size (10.25 × 15.5 × 13.7) to be introduced into the home cages of rodents and are linked to a monitoring system (Hub) that runs on batteries and can register the activity through a set of programs (Wheel Manager Software). As mentioned above, all experimental mice were group-housed. However, mice in the exercise condition were individually placed in a plastic cage different from their home cage with a low-profile running wheel for 1h three times a week (Monday, Wednesday and Friday). In Study 2, mice in the physical exercise condition had access to the running wheel throughout the experimental procedure and the socially defeated weeks had access immediately before each session. In contrast, in Study 4, mice in the physical exercise condition had access to the running wheel only between PND 26 and 46, prior to the initiation of the social defeat sessions.

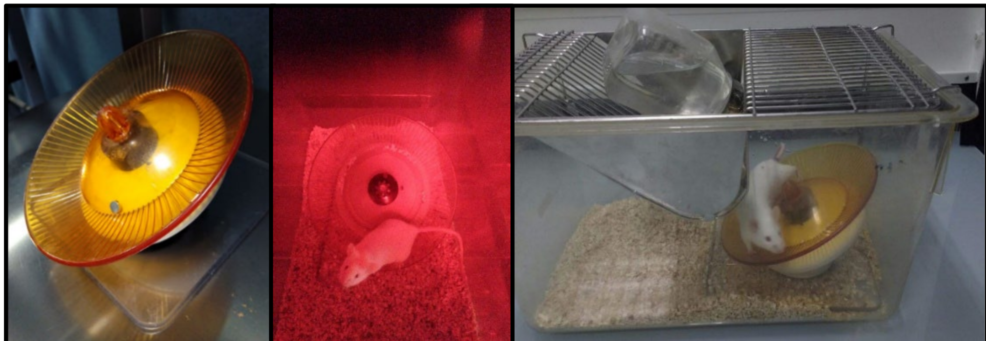

Figure 11. Voluntary Wheel Running. On the left, low-profile running wheel. In center and on the right, images of the placement of the wheels inside the cages.

3.6.3. Environmentally enriched housing

In Study 3, mice in the regular housing condition were housed in groups of four in transparent plastic cages (27 × 27 × 14 cm) with no more enrichment than standard bedding (wood flakes 1–3.35 mm), nesting material (paper strands) and two wooden gnaw sticks (5 x 1 × 1 cm) per cage. Male mice in EE conditions were housed in groups of four in plastic cages (59 x 38 × 20 cm) with standard bedding and nesting material, two wooden gnaw sticks plus additional PVC tunnel (13 × 5.5 cm) and a plastic mouse house (12.5 x 10.5 × 11 cm; Giménez-Gómez et al., 2021).

Figure 12. Environmental enrichment cage

3.6.4. Stress inoculation protocol

In study 5, half of the experimental groups were subjected to a single session of SD on PND 28. This agonistic encounter was conducted following the same protocol as the SD detailed below in section 3.3. The only difference is that only one encounter was conducted during adolescence stage.

3.7. Drinking in the dark

In Studies 5, 6 and 7, the Drinking in the Dark (DID) paradigm was implemented as a pre-exposure phase to ethanol before starting the oral ethanol SA procedure. Following the basic paradigm of Rhodes et al. (2005), the test consists of two phases.

The first is habituation, where the animals are removed from their cages to be housed individually for one week to habituate them to the cages and the suction tubes containing a ball bearing at the end to prevent leakage, which will be used throughout the test. In the second phase of the protocol, the test begins 3 hours after lights out and the water bottles are replaced with 10 ml graduated cylinders containing a 20% (v/v) ethanol solution. These will remain in place for 2 h. After this 2-h period, the animals are returned to their grouped cages, with food and water bottles *ad libitum* again. This procedure is repeated on days 2 and 3, and on day 4, the procedure lasts for 4 h. In addition, immediately after each day, liquid consumption is recorded. Fresh ethanol solution is prepared each day. In our case, we will maintain the protocol for two consecutive weeks, one for habituation and one for testing.

3.8. Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer.

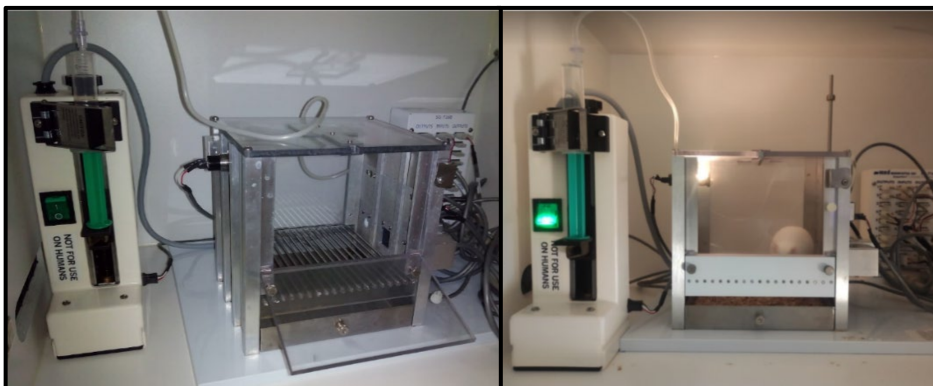

Figure 13. Modular operant chambers.

In Studies 1, 2, 3 and 4, active nose-pokes delivered 36 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% ethanol consumption.

Training phase (8 days): Two days before the initiation of the experiment, access to the standard diet was restricted to 1h per day. Before the first training session, water was withdrawn for 24h, and food allotment was provided 1h prior to the session to increase the motivation for active nose-poking. During the subsequent three days, water was provided ad libitum, except during the 1h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1h after the end of each daily session and water was available ad libitum to avoid ethanol consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 µl of 0.2% (w/v) saccharin reinforcement.

Saccharin substitution (9 days): The saccharin concentration was gradually decreased as the ethanol concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% ethanol; 0.10% Sac −4% ethanol; 0.05% Sac −6% ethanol).

6% ethanol consumption (11 days): The aim of the last phase was to evaluate the number of active nose-poke responses, the 6% ethanol (v/v) intake and the motivation to drink. This phase began 38 days after the last SD. After each session, the alcohol that remained in the receptacle was collected and measured with a

micropipette. To achieve this goal, during the last phase, the number of active responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, FR3 (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a PR session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40. All the sessions lasted 1 h, except the PR session, which lasted 2 h (Navarrete et al., 2012, 2014).

The following modifications were made in studies 5, 6 and 7. Food and water deprivation was deleted. Therefore, the saccharin substitution phases were deleted. Mice began the training phase directly exposed to 20% ethanol solution and active nose-pokes delivered 20 µl of solution. This training phase lasted approximately 12 days. Subsequently, the FR1 schedule was initiated for 10 days (the FR3 schedule was deleted). Finalizing the procedure with the PR schedule. The duration time of each phase, the environmental cues and the operation of the boxes were maintained as in the original protocol.

3.9. Immunoassay analysis

To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and cerebral structures dissected with a

brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Franklin & Paxinos, 2008), which were then kept in dry ice until storage at −80 °C. Before IL-6, CX3CL1, CXCL12 and BDNF determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of IL-6, CX3CL1, CXCL12 and BDNF in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates. All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the final results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of picograms of IL-6, CXCL12 and BDNF, and nanograms of CX3CL1. Data are expressed as pg/mg or ng/mg of protein for tissue samples.

3.10. Immunohistochemistry and morphometric digital image analysis

In Study 7, two 40 µm-thick coronal brain sections were randomly selected from six mice per group. The sections were washed three times in PBS and immersed in a solution of 3% (v/v) normal horse serum plus 0.5% (v/v) Triton X-100 in PBS for 3 h at 4°C under agitation to permeabilize and block nonspecific sites. The sections

were then incubated with the following primary antibodies diluted in a solution of 1% (v/v) normal horse serum and 0 3% (v/v) Triton X-100 in PBS: mouse anti-MAP-2 (1:1.000, Sigma-Aldrich, Cat# M4403, RRID:AB_477193), mouse anti- NF200 (1:1.000, Sigma-Aldrich, Cat# N0142, RRID:AB_477257), mouse anti- S-100β (1: 1.000, Sigma-Aldrich, Cat# S2532, RRID:AB_477499), rabbit anti-GFAP (1:3.000, Agilent Cat# Z0334, RRID:AB_10013382).

The sections were incubated at 4°C overnight under agitation. After three washes in PBS, sections were incubated for 3 hours in the dark with fluorescent secondary antibodies: Alexa FluorTM 568-conjugated anti-mouse IgG (1:1.000, Invitrogen, Cat# A11004, RRID:AB_143162) and Alexa FluorTM 488-conjugated anti-rabbit IgG (1:1.000, Invitrogen, Cat# A11008, RRID:AB_143165). Sections were subsequently counterstained with Hoechst 33342 (1:1.000, Sigma-Aldrich) to label nuclei, mounted on gelatin-coated slides and covered with 70% glycerol mounting medium.

Photographs were taken in an inverted Olympus IX83 microscope with an objective of 20×. Images were acquired using high-resolution digital monochromatic sCMOS Orca camera (Hamamatsu) and CellSens Dimension CS-DI-V1 software. All measurements were performed with ImageJ software (NIH1). From immunostaining, the percentage of area covered by MAP-2- and NF200-positive fibers was measured, as well as the optical intensity of S-100β and GFAP expression in 20× primary magnification images.

3.11. Statistics

In Studies 3, 4, 5, 6 and 7, mice were classified into resilient and susceptible groups based on the SIT, wich is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on

the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups.

To analyze SD sessions, DID procedure and acquisition of ethanol SA, a repeated-measures ANOVAs were performed. Moreover, to analyze EPM, PR schedule, neuroinflammatory response, BDNF and immunochemistry results, one-way ANOVAs were performed.

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. Statistical analyses were performed using SPSS Statistics (v24 or v.26; IBM, NY, USA) for behavioral data and GraphPad Prism (v8; GraphPad Software Inc., CA, USA) for graph design. Data were expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant.

4. RESULTS

Study 1

Oxytocin reverses ethanol consumption and neuroinflammation induced by social defeat in male mice

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(Annex 1)

Abstract

Oxytocin (OXT) modulates social interactions, attenuates stressful responses and can decrease drug-seeking and taking behaviors. In previous studies, we observed that social defeat (SD) induced a long-lasting increase in ethanol intake and neuroinflammation in male mice. We also know that OXT blocks the increase in cocaine reward induced by SD. Therefore, in the present study we aimed to evaluate the effect of 1 mg/kg of OXT administered 30 min before each episode of SD on ethanol consumption and the neuroinflammatory response in adult male mice. Three weeks after the last SD, mice underwent oral ethanol self-administration (SA) procedure, and striatal levels of the two chemokines CX3CL1 and CXCL12 were measured after the last SD and at the end of the ethanol SA. OXT administration blocked the increase in voluntary ethanol consumption observed in defeated mice, although it did not affect motivation for ethanol. An increase in the striatal levels of CX3CL1 and CXCL12 was observed in defeated animals immediately after the last defeat and after the ethanol SA. However, defeated mice treated with OXT did not show this increase in the neuroinflammatory response. In conclusion, OXT treatment can be a powerful therapeutic target to reduce the negative effects of social stress on ethanol consumption and the neuroinflammatory process.

Keywords: Chemokines; Ethanol; Neuroinflammation; Oxytocin; Self-administration; Social defeat; Stress.

Abbreviations:

BBB: blood-brain barrier; CNS: central nervous system; CPP: conditioned place preference; DA: dopamine; EtOH:ethanol; FR1: fixed ratio 1;FR3: fixed ratio 3; HPA: hypothalamic-pituitary-adrenal axis; NAcc: nucleus accumbens; OXT: oxytocin; PR: progressive ratio; SA: self-administration; SD: social defeat; VTA: ventral tegmental area

1. Introduction

Oxytocin (OXT) is a nonapeptide produced in the supraoptic and paraventricular nuclei of the hypothalamus and is released into blood circulation through neurohypophysis. OXT not only acts as a modulating hormone of physiological functions such as uterine contractions and lactation (Leng et al., 2015; Russell et al., 2003), but also as a neuromodulator in the central nervous system (CNS), since it is involved in maternal (Kim & Strathearn, 2016; Marlin et al., 2015), reproductive (Veening et al., 2015) and social (Caldwell, 2017; Caldwell et al., 2017) behaviors. Clinical and preclinical studies have demonstrated that OXT has a modulating role in a wide variety of social interactions that have been relevant in the evolution of mammals, as in social recognition, sexual behavior, pairing, parental behavior and aggression (Ebert & Brüne, 2018; Chen et al., 2017; Lazzari et al., 2019; Lukas et al., 2011; Stohn et al., 2018). In addition, OXT has an anxiolytic effect and attenuates the hypothalamic-pituitary-adrenal (HPA) axis response to stress (Winter & Jurek, 2019; Yang et al., 2019). OXT also produces a reduction in amygdala functions and, therefore in stress reactivity (Kirsch et al., 2005; Labuschagne et al., 2010; Lukas et al., 2011; Nasanbuyan et al., 2018; Onaka et al., 2012). OXT produces an increase in the connectivity between the amygdala and the medial rostral prefrontal cortex, which plays a critical role in social cognition (Sripada et al., 2013). This action points to OXT as a therapeutic target in fear and social disorders such as post-traumatic stress disorder, generalized anxiety disorder or social anxiety disorder (Neumann & Landgraf, 2012; Steinman et al., 2019; Wang, L. et al., 2018).

In recent years, several studies have shown that administration of OXT reduces the effects caused by social stress, promoting an increase in social interaction and decreasing the anhedonia and social avoidance that characterizes social stress (Borland et al., 2018; Ebert & Brüne, 2018; Ferrer-Pérez et al., 2019; Lukas et al., 2011; Nasanbuyan et al., 2018; Steinman et al., 2016; Wang, L. et al., 2018). The most commonly used social stress model is social defeat stress (SD), based on the

resident-intruder paradigm. This model evaluates avoidance/flee and defensive/submissive behaviors in experimental mice caused by the territoriality of aggressive residents (Covington III & Miczek, 2001; Miczek et al., 2004). Animal models of SD stress are known to produce long-term consequences, such as anhedonia, decreased social interaction, anxiety, depression, or drug addiction (Burke & Miczek, 2014; Liu et al., 2017; Miczek et al., 2008; Shimamoto et al., 2015).

It is well known that social stress influences the development of addiction, increasing drug-seeking and taking behaviors, withdrawal and relapse (Koob & Schulkin, 2019; Montagud-Romero et al., 2018; Ruisoto & Contador, 2019). Rodents exposed to SD stress increase consumption, preference and motivation for ethanol (EtOH) in different paradigms, such as EtOH-induced conditioned place preference (CPP) (Karlsson et al., 2017; Macedo et al., 2018), the two-bottle choice (Croft et al., 2005; Deal et al., 2018; Newman et al., 2018) or drinking in the dark (Caruso et al., 2018). In oral self-administration (SA), our and other research groups described a significant increase of EtOH consumption and a greater motivation to obtain EtOH in socially defeated mice (Norman et al., 2015; Reguilón et al., 2020; Rodríguez-Arias et al., 2016; Van Erp & Miczek, 2001). It must also be noted that OXT administration decreases drug use, withdrawal symptoms, and drug-seeking behaviors associated with various drugs of abuse (Leong et al., 2018; Pedersen, 2017). Specifically, OXT administration reduces EtOH consumption in rodents (King et al., 2017; MacFadyen et al., 2016; Peters et al., 2017; Stevenson et al., 2017; Tunstall et al., 2019). The administration of OXT would normalize brain changes induced by drugs of abuse, thereby reducing consumption (King et al., 2020; Leong et al., 2018). Tunstall and co-workers (2019) proposed that OXT, by blocking GABA signaling at the central nucleus of the amygdala, decreases excessive alcohol consumption in alcohol-dependent rats. Other studies suggest that OXT acting on the mesolimbic pathway modifies dopaminergic signaling (Borland et al., 2018;

Peris et al., 2017; Weber et al., 2018). In fact, subchronic administration of OXT decreases the basal release of dopamine (DA) in the nucleus accumbens (NAcc) (Estes et al., 2019). Additionally, intracerebroventricular OXT administration blocks the DA-release induced by the administration of EtOH in rats (Peters et al., 2017).

In the last decades, neuroinflammatory processes have been widely studied in addiction and stress-related disorders (Calcia et al., 2016; Northrop & Yamamoto, 2013; Orio et al., 2019; Zhang et al., 2018). After exposure to SD, there are reports of detriment in blood-brain barrier (BBB) permeability (Rodríguez-Arias et al., 2017) and an increase in cytokines (Ferrer-Pérez et al., 2018; Wohleb et al., 2011, 2012, 2014) and chemokines levels (Reguilón et al., 2020; Rossetti et al., 2016; Wohleb et al., 2013). Moreover, SD-induced neuroinflammation can exacerbate the well-known brain damage produced by EtOH (Karlsson et al., 2017; Montesinos et al., 2016; Pascual et al., 2015; Zhang et al., 2018). Interestingly, some recent studies have shown that OXT treatment mitigates the neuroinflammatory response in mice caused by maternal separation (Amini-Khoei et al., 2017) or post-traumatic stress (Wang, S. et al., 2018).

There are no studies evaluating the potential effect of OXT on the increased consumption of EtOH induced by social defeat. However, we know that mice treated with OXT prior to each SD did not develop the long-lasting increase in cocaine-induced CPP (Ferrer-Pérez et al., 2019). Therefore, in the present study we aimed to evaluate the effect of 1 mg/kg of OXT administered 30 min before each SD episode on the long-lasting increase in EtOH consumption and the neuroinflammatory response. After three weeks of the last SD encounter, we assessed the consumption and motivation for EtOH using the EtOH-SA oral paradigm. Furthermore, we also evaluated the levels of chemokines CX3CL1 and CXCL12 in the striatum after the last exposure to SD and at the end of the EtOH-SA procedure.

2. Material and methods

2.1 Subjects

105 male OF1 mice (Charles River, France) were delivered to our laboratory at 42 days of age. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998) (n = 15 adult mice). All mice were housed under the following conditions: constant temperature, a reversed light schedule (lights off at 08:00 and on at 20:00), and food and water were freely available ad libitum, except during the behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committee (University of Valencia).

2.2 Drugs

For the oral SA procedure, absolute EtOH (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) EtOH solution equivalent to a 7.6% (v/v) EtOH solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water. Before SD, mice were injected 1 mg/kg of oxytocin (Sigma-Aldrich, Madrid, Spain) in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs.

2.3 Experimental design

The experimental design is depicted in Fig. 1. The first set of mice, composed by 40 mice, were employed to evaluate the effect of OXT on the neuroinflammation response induced by SD. Half of the mice received an i.p. injection of saline or 1 mg/kg of OXT 30 min before each defeat procedure. Half of the mice in each group were sacrificed immediately after the first social defeat, while the remainder were sacrificed after the fourth defeat. The exploration group (CON) treated with saline

30 min before the procedure was sacrificed after the 1st exploration. The striatum was collected for further biochemical analysis.

The second set of mice was composed of a total of 50 mice that underwent an SD/CON protocol. Similarly, half of the mice received an i.p. injection of saline or 1 mg/kg of oxytocin 30 min before each exploration or defeat procedure. Subsequently, three weeks after the last defeat, the mice initiated the EtOH SA protocol for approximately 28 days. At the end of this test, all the mice were sacrificed to obtain the striatum for further analysis of the chemokine levels (8 mice of each experimental group).

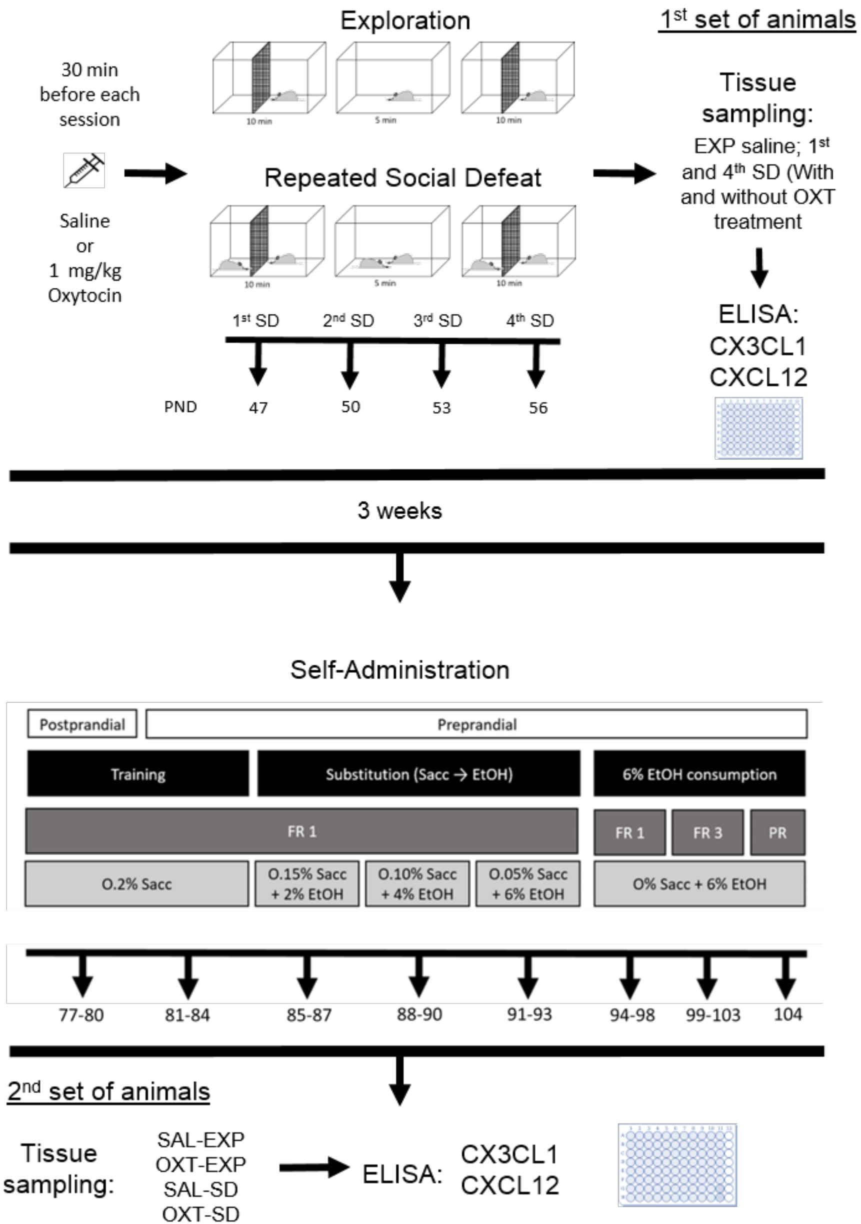

Fig. 1. Experimental design. All the mice were divided into four experimental groups: SAL-CON, SAL-SD, OXT-CON and OXT-SD. Saline or 1 mg / kg OXT were injected 30 min before each exposure to exploration (CON) or SD. Four sessions were carried out every 72 h

of SD or exploration in the corresponding groups. A 1st set of mice was sacrificed to carry out the biochemical analyses 4 h after the 1st exposure to the exploration, 8 mice were sacrificed and, in the groups exposed to stress, the sacrifice and extraction of samples were performed 4 h after the 1st and 4th exposures to SD (8 mice per group). After finishing the encounters, the rest of the mice were kept without manipulation for 3 weeks, and then the protocol of oral EtOH SA began. This procedure lasted 28 calendar days and 24 h after the end of the PR schedule, the mice were sacrificed to obtain the samples for their subsequent biochemical analysis (2nd set of mice).

2.4 Repeated social defeat encounters

Animals in the stress/defeated groups were exposed to 4 episodes of SD lasting 25 min each on postnatal days (PND) 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the aggressive resident (Covington III & Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each social defeat protocol was video-recorded and ethologically analysed. Threat and attack behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was replaced for a further 10 min to allow social threats from the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, after which the behavioral tests began.

In the corresponding groups, physiological saline or oxytocin was administered 30 min before each social encounter or exploration (control groups).

2.5. Apparatus and procedures: Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral EtOH SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-poke delivered 36 µl of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6 s time-out period. The inactive nose-poke did not produce any consequence.

To evaluate the consequences of SD (with or without oxytocin) on the acquisition of oral EtOH SA, animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% EtOH consumption.

2.5.1. Training phase (8 days)

Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and food allotment was provided 1 h prior to the session to increase the motivation for active nose-poking. During the subsequent 3 days, water was provided ad libitum, except during the 1 h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1 h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 µl of 0.2% (w/v) saccharin reinforcement.

2.5.2. Saccharin substitution (9 days)

The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% EtOH; 0.10% Sac −4% EtOH; 0.05% Sac −6% EtOH).

2.5.3. 6% ethanol consumption (11 days)

The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 6% EtOH (w/v) intake and the motivation to drink. This phase began 38 days after the last social defeat. After each session, the alcohol that remains in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of effective responses and EtOH consumption (µl) were measured under fixed ratio 1 (FR1) for 5 daily consecutive sessions, fixed ratio 3 (FR3) (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement, according to the previous scale (for example, if an animal activates the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement). Therefore, the breaking point value for this animal would be 40. All the sessions lasted 1 h, except the PR session, which lasted 2 h.

2.6. Tissue sampling

Striatum samples were taken 4 h after the first exploration, the first and the fourth agonistic encounters and a final sample was obtained after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and the striatum dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Paxinos & Franklin, 2001), which were then kept in dry ice until storage at −80 °C. Before CX3CL1 and CXCL12 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al., 2010. Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

2.7. Determination of CX3CL1 and CXCL12 levels

To determine CX3CL1 and CXCL12 concentration on tissues, we used a Mouse CX3CL1 ELISA Kit obtained from Abcam (Ref: ab100683) and a Mouse CXCL12 Kit obtained from Abcam (Ref: ab100741), which were performed following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450 nm and final results were calculated using a standard curve following the manufacturer's instructions, and were expressed as ng/mg for CX3CL1 and as pg/mg for CXCL12 (tissues).

2.8. Statistical analysis

The data of the ethological analysis of resident and intruder mice were analysed by a two-way ANOVA with a one between-subjects variables – Treatment (with or

without OXT)- and a one within variable - SD encounter- with two levels: first and fourth SD.

To analyse acquisition of EtOH SA, a three-way ANOVA was performed with two between-subjects variables –Stress (CON or SD) and Treatment (saline or OXT)– and a within-subjects variable in both cases –Days, with five levels of FR1 or FR3– . The effects of SD and treatment on breaking point values and EtOH consumption during PR was analysed by a two-way ANOVA, with two between-subjects variable –Stress (CON or SD) and Treatment (Saline and OXT).

Data related to chemokine concentrations after SA procedure were analysed using a two-way ANOVA, with two between-subjects variables –Stress (CON or SD) and Treatment (Saline and OXT). For the striatal chemokine concentrations after the SD, a one-way ANOVA was employed with a between variable –Group (CON, 1st SAL-SD, 4th SAL-SD, 1st OXT-SD, 4th OXT-SD).

All ANOVAs were followed by a Bonferroni's post-hoc test and partial eta-square ($\eta 2p$) was performed to calculate effect sizes. Moreover, Cohen's d effect sizes were calculated for all statistically different pair-wise comparisons. The results are reported as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. OXT treatment does not affect the ethological behavior of repeated SD

With regard to intruder mice, the ANOVA revealed a significant difference in the variables Day for Total Time [$F(1, 18) = 8.246$; $p = 0.01$; $\eta 2p = 0.314$] and Day for Mean Time [$F(1, 18) = 15.179$; $p = 0.001$; $\eta 2p = 0.457$] in Avoidance/Flee behavior. All mice spent less total ($p = 0.01$, $d = 0.8880$) and mean ($p = 0.001$, $d = 1.3916$) time in Avoidance/Flee behavior. Moreover, the ANOVA revealed a significant difference in the variables Day for Total Time [$F(1, 18) = 9.784$; $p < 0.01$; $\eta 2p = 0.352$] and Day for Latency [$F(1, 18) = 6.799$; $p < 0.05$; $\eta 2p = 0.274$] in

Submission/Defensive behavior (Table 1). All mice spent more time in Submission/Defensive behavior in the fourth SD compared with the first SD ($p < 0.01$, $d = 1.2751$,) and showed a higher latency of this behavior in the first SD compared to the fourth SD ($p < 0.05$, $d = 0.8415$).

			Treated with Saline		Treated with Oxytocin	
		Encounters	1st	4th	1st	4th
Intruder mice	Avoidance/ Flee	Time (s)	22±2	16±1**	27±7	16±2**
	Defense/ Submission	Time (s)	21±3	40±2**	25±7	43±5**
Resident mice	Threat	Time (s)	13±1	9±1*	7±1+++	7±1
	Attack	Time (s)	10±1	29±3***	11±6	30±3***

Table 1. Ethological analyses of social defeat. Behavior of resident and intruder mice during 5-min agonistic encounters. p < ***, p < **, p < * significant differences between the 1st and 4th SDs. p < +++, p < ++, p < + significant differences between SAL-treated and OXT-treated mice.

In the behavior of the resident mice, the ANOVA revealed a significant difference in the variable Treatment for Mean Time [$F(1, 18) = 6.211$; $p < 0.05$; $\eta 2p = 0.257$] and in the interactions Day × Treatment for Total Time [$F(1, 18) = 4.731$; $p < 0.05$; $\eta 2p = 0.208$], and Day × Treatment for Latency [$F(1, 18) = 5.925$; $p < 0.05$; $\eta 2p = 0.248$] in Threat behavior (Table 1). The post-hoc comparison showed that resident mice spent less mean time threating OXT-treated mice ($p < 0.05$, $d = 0.8645$), and resident mice spent less time threating OXT-treated mice during the first SD ($p < 0.001$, $d = 3.8923$), but no difference was observed in the last SD. In addition, resident mice showed more threat behavior to saline-treated mice during the first SD than the fourth ($p < 0.05$, $d = 3.04539$). Finally, resident mice showed a higher latency of threat from SAL-treated mice in the first SD compared to fourth

SD ($p < 0.05$, $d = 1.1711$) and showed a higher latency of threat from SAL-treated mice in the first SD compared to OXT-treated mice ($p = 0.001$, $d = 1.8749$). Regarding attack behavior, the ANOVA showed an effect of the variables Day for Total Time [$F(1, 18) = 32.331$; $p < 0.001$; $\eta 2p = 0.642$] and Day for Mean Time [$F(1, 18) = 5.872$; $p < 0.05$; $\eta 2p = 0.246$], and in the interaction Day × Treatment for Latency [$F(1, 18) = 5.925$; $p = 0.01$; $\eta 2p = 0.315$]. Resident mice showed more total ($p < 0.001$, $d = 1.74268$) and mean ($p < 0.05$, $d = 0.7357$) time in attack behavior in the last SD compared to the first. On the one hand, resident mice showed a higher latency of attack behavior with the SAL-treated mice during the first SD compared to the fourth SD ($p = 0.001$, $d = 0.9259$). On the other hand, they showed a higher latency of attack behavior with SAL-treated mice compared to OXT-treated mice during the first ($p < 0.01$, $d = 1.3989$) and fourth ($p < 0.05$, $d = 1.1589$) SDs.

3.2. Oxytocin attenuates the increase in oral ethanol SA induced by social stress

No differences were found between the animals during training or substitution phases, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of effective responses during the FR1 schedule of EtOH SA revealed a significant effect of the interaction Days × Treatment [$F(4, 184) = 3.488$; $p < 0.01$; $\eta 2p = 0.070$] and Stress × Treatment [$F(1, 46) = 6.301$; $p < 0.05$; $\eta 2p = 0.120$] (Fig. 2a). Post-hoc comparison showed that mice treated with oxytocin showed fewer effective responses compared to non-treated mice on days 1 ($p < 0.05$, $d = 0.68580$), 2 ($p < 0.001$, $d = 1.17522$) and 3 ($p < 0.01$, $d = 1.05017$). Moreover, effective responses were higher in the saline-treated defeated group (SAL-SD) with respect to the rest of the groups (p's < 0.001 in all cases; $d = 2.248$ for SAL-CON group, $d = 2.542$ for OXT-CON group, $d = 1.487$ for OXT-SD group). With respect to EtOH consumption, the ANOVA revealed a significant effect of the interaction Stress × Treatment ([$F(1, 46) = 12.678$; $p = 0.001$; $\eta 2p = 0.217$]) (Fig. 2b). The post-hoc comparison showed that the SAL-SD group consumed EtOH at

higher rates than the rest of the groups (p's < 0.001 in all cases; d = 1.7045 for SAL-CON group, d = 1.7697 for OXT-CON group, d = 1.1609 for OXT-SD group).

During the FR3 schedule, the ANOVA revealed a significant effect of the variable Stress [$F(1, 46) = 6.866$; $p < 0.05$; $\eta 2p = 0.130$] and the interaction Days × Treatment [$F(4, 184) = 4.940$; $p = 0.001$; $\eta 2p = 0.097$] for the number of effective responses (Fig. 2a). Defeated groups (SAL-SD and OXT-SD) showed higher number of effective responses than non-stressed groups ($p < 0.05$; d = 0.664). Moreover, mice treated with oxytocin (OXT-CON and OXT-SD) showed a lower number of effective responses on day 8 compared to day 9 ($p = 0.0$; d = 0.507) and saline-treated groups (SAL-CON and SAL-SD) showed a lower number of effective responses on day 6 compared to days 7 ($p < 0.01$; d = 0.576) and 8 ($p < 0.05$; d = 0.509). With respect to EtOH consumption, the ANOVA revealed a significant effect of the variable Stress [$F(1, 46) = 5.084$; $p < 0.05$; $\eta 2p = 0.100$] and the interaction Days × Treatment [$F(4, 184) = 6.308$; $p < 0.001$; $\eta 2p = 0.121$] (Fig. 2b). Defeated groups (SAL-SD and OXT-SD) consumed significantly more EtOH than non-stressed groups ($p < 0.05$; d = 0.5476). Also, all animals treated with oxytocin (OXT-CON and OXT-SD) showed significantly higher consumption rates on day 6 ($p < 0.05$; d = 0.666) and a significantly lower consumption rate on day 8 ($p < 0.05$; d = 0.644) with respect to saline-treated groups.

During the PR, the ANOVA for the breaking point values of EtOH SA revealed a significant effect of the variable Stress [$F(1, 46) = 4.769$; $p < 0.05$; $\eta 2p = 0.094$] (Fig. 2c). The post-hoc comparison showed that the breaking point values were higher in the defeated groups (SAL-SD and OXT-SD) with respect to the non-stressed groups ($p < 0.05$; d = 0.6227). The ANOVA for EtOH consumption during PR did not reveal any significant effect (Fig. 2d).

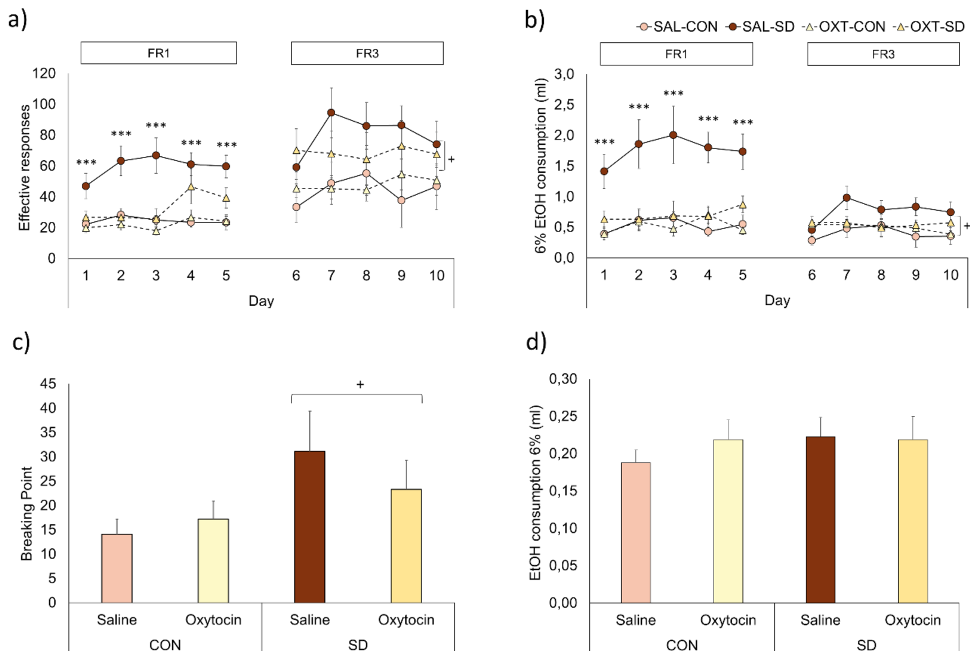

Fig. 2. Effects of oxytocin treatment during SD procedure on the increase in oral EtOH self-administration induced by social stress in OF1 mice. Mice were divided in the following four treatment groups: CON mice were allowed to explore a new cage and were treated with saline (SAL-CON, n = 14) or with oxytocin (OXT-CON, n = 12); The SD group was exposed to social defeat and treated with saline (SAL-SD, n = 12) or with oxytocin (OXT-SD, n = 12). The dots represent means and the vertical lines ± SEM of (a) the number of effective responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent means and the vertical lines ± SEM of (c) the breaking point values and (d) the volume of 6% EtOH consumption during PR. ***$p < 0.001$ significant difference with SAL-CON, OXT-CON and OXT-SD; +$p < 0.05$ significant difference with defeated groups (SAL-SD, OXT-SD) with respect to non-defeated groups (SAL-CON, OXT-CON).

3.3. OXT blocked the increase in CX3CL1 and CXCL12 striatal levels induced by SD

The ANOVA revealed a significant effect of the variable Group [$F(4, 35) = 9.055$; $p < 0.001$; $\eta 2p = 0.509$] for the striatal levels of CX3CL1 (Fig. 3a). The post-hoc comparison showed that defeated animals treated with saline (SAL-SD) showed higher CX3CL1 levels after the 4th defeat than those of the exploration condition (SAL-CON) ($p < 0.001$; $d = 1.8313$). In addition, after the 4th SD encounter, the SAL-SD group showed a significantly higher striatal level of CX3CL1 than the OXT-SD group ($p < 0.001$; $d = 2.1480$).

With respect to the striatal protein levels of CXCL12, the ANOVA showed a significant effect of the variable Group [$F(4, 35) = 11.874$; $p < 0.001$; $\eta 2p = 0.576$] (Fig. 3b). The post-hoc comparison showed that the SAL-SD group showed higher CXCL12 levels after the 4th defeat compared to the SAL-CON group ($p < 0.01$; $d = 1.997$). After the 1st and 4th SD encounters, the SAL-SD group showed higher CXCL12 levels than the OXT-SD group ($p < 0.05$; $d = 1.551$ and $p < 0.001$; $d = 2.979$, respectively).

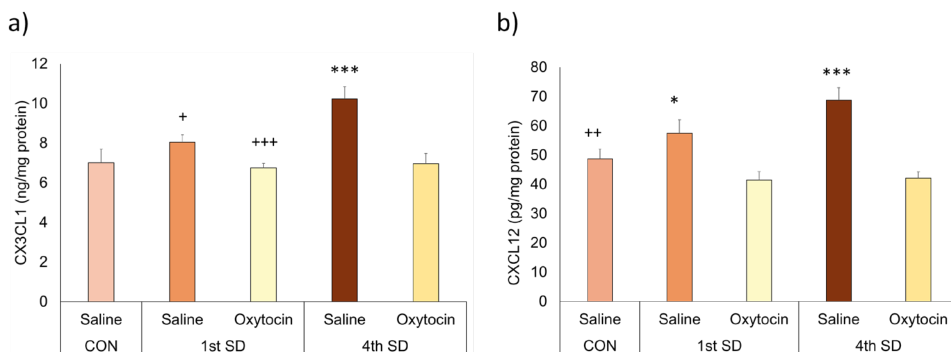

Fig. 3. Striatal levels of CX3CL1 and CXCL12 after SD. Concentration of (a) CX3CL1 and (b) CXCL12 after exploration condition (CON), 1st defeat in mice treated with saline (1st SAL-SD) or oxytocin (1st OXT-SD) and 4th defeat in mice treated with saline (4th SAL-SD) or oxytocin (4th OXT-SD). The columns represent means and the vertical lines ± SEM

of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice (n = 8 in all groups). ***p < 0.001, **p < 0.01 differences with respect to the SAL-CON group; +++p < 0.001, +p < 0.05 differences with respect the corresponding SAL-SD group.

3.4. OXT blocked the increase in CX3CL1 and CXCL12 striatal levels observed in defeated mice after oral ethanol SA

The ANOVA for CX3CL1 and CXCL12 striatal levels showed a significant effect of the interaction Stress × Treatment [$F(1, 28) = 20.019$; $p < 0.001$; $\eta 2p = 0.417$] (Fig. 4a) and [$F(1, 28) = 4.686$; $p < 0.05$; $\eta 2p = 0.143$] (Fig. 4b). The post-hoc comparison revealed that defeated animals treated with saline (SAL-SD) showed higher concentrations of these chemokines compared to the rest of the groups (p's < 0.001; d = 2.845 in SAL-CON group, d = 3.051 in OXT-CON group and d = 3.317 in OXT-SD group for CX3CL1 and p's < 0.01; d = 2.160 in SAL-CON group, d = 1.584 in OXT-CON group and d = 1.351 in OXT-SD group for CXCL12).

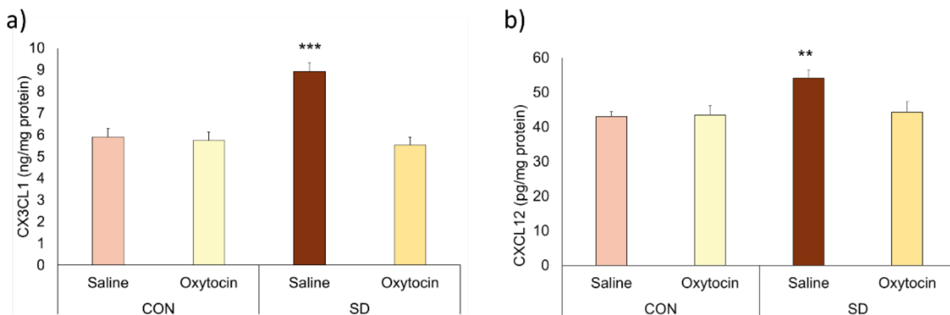

Fig. 4. Striatal levels of CX3CL1 and CXCL12 after EtOH SA. CX3CL1 (a) and CXCL12 (b) protein levels after oral EtOH SA in the following treatment groups: CON group allowed to explore a new cage and treated with saline (SAL-CON, n = 8) or with oxytocin (OXT-CON, n = 8); and SD group exposed to social defeat and treated with saline (SAL-SD, n = 8) or with oxytocin (OXT-SD, n = 8). The columns represent means and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice. ***p < 0.001; **p < 0.00 significant differences with the rest of the groups.

4. Discussion

Our results showed for the first time that OXT administration before each SD blocked the increase in EtOH intake induced by SD. OXT treatment also reduced the neuroinflammation response measured by the striatal levels of the chemokines CX3CL1 and CXCL12 immediately after SD and at the end of the oral EtOH-SA. However, OXT treatment failed to reduce the motivation to obtain EtOH in the PR schedule.

As OXT was administered prior to each SD, it was critical to corroborate that the behavior of the intruder and the resident mice did not vary due to the OXT administration. As we have previously shown (Ferrer-Pérez et al., 2019), treatment with OXT prior to each SD does not alter the behavior of the intruder mice. OXT-treated mice spent similar amounts of time in avoidance/flee and defence/submission behaviors as SAL-treated mice. During the first defeat, resident mice threatened OXT-treated intruders for a shorter time compared to the saline group, differences that disappeared in the last SD. In addition, no differences in attack behavior were observed depending on the treatment. Although SD was effectively experienced by animals treated with OXT, we cannot rule out differences in the way OXT-treated animals coped with the SD experience.

The current study corroborates that SD produces a long-lasting increase in EtOH intake using the oral EtOH-SA paradigm (Caldwell & Riccio, 2010; Norman et al., 2015; Reguilón et al., 2020; Rodríguez-Arias et al., 2016). In the present study, we observed that defeated SAL-treated mice consumed more EtOH and showed a higher number of effective responses compared to SAL-treated, non-stressed animals during the FR1 schedule. Conversely, defeated OXT-treated mice consumed significantly less EtOH and showed fewer effective responses. To our knowledge, there are no studies evaluating the role of OXT on the increase in the oral EtOH-SA paradigm induced by SD. Several studies have established that OXT treatment

reduces EtOH consumption (King et al., 2017; MacFadyen et al., 2016; Peters et al., 2017), and operant responses in rodents (Tunstall et al., 2019). These results suggest that OXT can modulate the DA pathway projecting from the hypothalamus onto the ventral tegmental area (VTA) and the NAcc, which present OXT receptors (Grinevich et al., 2016). OXT targets VTA DA neurons, increasing extracellular DA in the NAcc, thereby increasing positive enhancements and signaling of natural rewards (Adinoff, 2004; Hung et al., 2017). Therefore, OXT stimulation in DA reward circuits can decrease EtOH-induced stimulation of DA neurons (Peris et al., 2020). However, in our experimental design, OXT was administered during SD, weeks earlier than the beginning of the EtOH exposure. Therefore, these direct mechanisms of OXT cannot explain our results. Moreover, OXT can potentially reduce corticosterone levels and normalize the HPA axis stress response (Peris et al., 2020). King and Becker (2019) observed that OXT attenuated the EtOH-seeking and reinstatement behavior caused by immediate stress (15 min before SA session).

The anxiolytic and anti-stress effects of OXT have been widely demonstrated (Bülbül et al., 2011; Grund et al., 2019; Krause et al., 2011; Peters et al., 2014; Smith et al., 2015). In this way, OXT could interfere in the experience of SD, decreasing the negative impact of stress. Supporting this hypothesis, we observed that a similar OXT administration than the one employed in this study can decrease the long-lasting increase in anxiety observed in socially defeated animals (Ferrer-Pérez et al., 2019). There is lack of consensus in the scientific literature about the effects of OXT on anxiety and stress. OXT can induce anxiolytic or anxiogenic effects modulating the salience of emotional contexts (see Jurek & Meyer, 2020), although the mechanisms are unclear (Shamay-Tsoory & Abu-Akel, 2016). The results observed vary considerably according to the dose used and the method of administration. Chronic OXT treatment increases the HPA response (Yoon & Kim, 2020), but acute administration only transiently increases corticosterone levels (Petersson et al., 1999). Intracerebroventricular infusions of low doses of OXT reduce the effects of

chronic stress, but high doses increase the anxiogenic behavior (Peters et al., 2014). However, infusions of OXT in the medial prefrontal cortex increase dopaminergic transmission, inducing an antidepressant effect in animals previously subjected to SD (Li et al., 2020). Important sex differences in the OXT modulation of stress effects have also been observed. While administration of OXT before SD produces an increase in the anxiogenic effects in females, a decrease is observed in males (Steinman et al., 2016; Steinman & Trainor, 2017).

Regarding the FR3 and PR schedules of the SA paradigm, a stress effect was obtained independent of OXT treatment. SAL- and OXT-treated defeated mice showed a greater effort to obtain a dose of EtOH during the FR3 schedule and showed a significantly higher breaking point during the PR schedule compared to non-stressed animals. The FR1 procedure assesses the potential liability of a drug and the consumption based on its unconditioned psychopharmacological effects (Sanchis-Segura & Spanagel, 2006). However, FR1 performance is less affected by incentive value or motivational factors. The activities of some neural systems (Salamone & Correa, 2002) or experimental conditions (Morgan et al., 2002) do not modify the number of reinforcers obtained under an FR1 schedule, resulting in significant behavioral changes when using progressive increases in ratio. Some studies associate an increased motivation (breaking point) during PR to DA release from the dorsolateral striatum (González-Marín et al., 2019). Dorsolateral striatum is involved in both the rewarding and motor effects of EtOH and specific aspects of incentive motivation (Chen et al., 2015), the signals that activate incentive salience in instrumental learning seem to be associated with DA activity in the dorsolateral striatum (González-Marín et al., 2019; Ostlund et al., 2011). Since the VTA dopaminergic system is extremely sensitive to stress (Anstrom et al., 2009; Krishnan et al., 2008; Miczek et al., 2008; Razzoli et al., 2011), one possible explanation could be that the OXT dose administered was incapable of blocking the increased motivation for EtOH induced by SD. Although many studies claim that OXT

administration reduces motivation in oral EtOH-SA (King et al., 2017; Tunstall et al., 2019), in these studies OXT is administrated immediately prior to the EtOH-SA paradigm.

SD induced an immediate and long-lasting increase in the striatal levels of both chemokines, CXC3L1 and CXCL12. Fractalkine or CX3CL1 has both an inflammatory and a proinflammatory function, and promotes microglial and astrocytic activation, proinflammatory cytokine secretion, ICAM-1 expression, and CNS T-cell recruitment during neuroinflammatory diseases (Galán-Ganga et al., 2019; Lauro et al., 2015; Lee et al., 2018). CXCL12 promotes the growth of neurites and neurogenesis (Jaerve & Müller, 2012; Opatz et al., 2009) but is also involved in cell migration that contributes to inflammation, attracting leukocytes through the BBB. Nevertheless, excessive production and accumulation of CXCL12 can be toxic and this inflammation can lead to brain damage. In the present study, we observed that the concentration level of both chemokines is higher after the 4th SD in defeated mice compared to non-stressed mice. Furthermore, we observed an anti-inflammatory effect of OXT, since defeated mice treated with OXT showed significantly lower levels of CX3CL1 after the first and fourth SD compared to the SAL-treated defeated mice. Similar results were observed with the striatal levels of CXCL12.

We have previously shown that SD induced a long-lasting increase in brain cytokines, as IL-6, (Ferrer-Pérez et al., 2018) and chemokines, as CX3CL1 and CXCL12, (Reguilón et al., 2020). Our results confirmed these findings and, more importantly, showed that prior administration of OXT blocked this neuroinflammatory response. There are no previous studies linking OXT administration and the neuroinflammatory response caused by SD. OXT has a short-term anti-inflammatory effect on the innate immune response (Bordt et al., 2019; Yuan et al., 2016), suppressing TNF-α, IL-1β, COX-2, and iNOS expression caused

by injection of liposaccharide bacteria (Inoue et al., 2019; Yuan et al., 2016). The protective effects of OXT have also been shown in rodents with brain damage or infection in which OXT induced an immediate decrease in the circulatory levels of proinflammatory cytokines and the infiltration of neutrophils in injured areas (Inoue et al., 2019; Yuan et al., 2016).

Moreover, these results were confirmed after the end of the EtOH-SA paradigm. Again, defeated mice showed higher levels of both chemokines when compared to non-stressed animals. Although exposure to EtOH is an important promoter of neuroinflammation (Pradier et al., 2018), we only observed a neuroinflammatory response after EtOH SA in the defeated group treated with saline. Saline non-stressed mice showed comparable levels of CX3CL1 and CXCL12 to those of the exploration group prior to EtOH SA exposure, non-exposed to stress or EtOH. This may be due to the fact that the concentration of EtOH used in SA is low (6%) and that the daily intake of non-stressed mice was not enough to induce neuroinflammation. On the other hand, defeated mice had higher EtOH intake and an elevated neuroinflammation response after SD, which in turn induced an increase of CX3CL1 and CXCL12 striatal levels. Stressed mice treated with OXT showed a significantly lower concentration of both chemokines than defeated mice treated with saline. This could be due to the same anti-inflammatory effect already shown after SD, but we have to bear in mind that, after SA, OXT-defeated mice consumed an amount of EtOH comparable to the amount consumed by saline non-stressed animals. Therefore, we have to take into consideration that the lack of neuroinflammatory response could be due to the lower EtOH intake.

5. Conclusions

In conclusion, our results suggest that OXT decreases the SD-induced increase in EtOH consumption. This effect could be due to a decrease in the neuroinflammatory response induced by SD, although other mechanisms cannot be ruled out, such as a

different coping with the defeat experience by the OXT-treated animals. These results point to OXT as a therapeutic target to reduce the negative effects of social stress on EtOH consumption and the neuroinflammatory process.

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Study 2

Voluntary wheel running protects against the increase in ethanol consumption induced by social stress in mice.

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Abstract

Previous studies have shown that exposure to social defeat (SD), a model of social stress, produces a long-term increase in the consumption of ethanol, most likely through an increase in the neuroinflammation response. The aim of the present study was to evaluate whether exposure to physical activity in the form of voluntary wheel running (VWR) could block the increase in ethanol consumption and the neuroinflammatory response induced by social stress. Mice were exposed to either 4 sessions of repeated social defeat (RSD) or a non-stressful experience. During the whole procedure, half of the mice were exposed to controlled physical activity, being allowed 1 h access to a low-profile running wheel three times a week. Three weeks after the last RSD, animals started the oral self-administration (SA) of ethanol (6% EtOH) procedure. Biological samples were taken 4 h after the first and the fourth RSD, 3 weeks after the last RSD, and after the SA procedure. Brain tissue (striatum) was used to determine protein levels of the chemokines fractalkine (CX3CL1) and SDF-1 (CXCL12). RSD induced an increase in ethanol consumption and caused greater motivation to obtain ethanol. The striatal levels of CX3CL1 and CXCL12 were also increased after the last RSD. VWR was able to reverse the increase in ethanol intake induced by social stress and the neuroinflammatory response. In conclusion, our results suggest that VWR could be a promising tool to prevent and reduce the detrimental effects induced by social stress.

Keywords: Social stress; Ethanol; Neuroinflammation; Chemokines; Physical exercise; Self-administration

Abbreviations:

RSD: repeated social defeat; SD: social defeat; EtOH: ethanol; SA: self-administration; FR1: fixed ratio 1; FR3: fixed ratio 3; PR: progressive ratio; TBC: two bottle choice; VWR: voluntary wheel running; HPA: hypothalamic-pituitary-adrenal; CPP: conditioned place preference; BBB: blood-brain barrier; PND: postnatal day

1. Introduction

Social stress is deeply implicated in the neural and behavioral alterations that contribute to the development of mental health disturbances and drug addiction (Beutel et al., 2018). Stressful experiences modify the reward system and are involved in the transition from drug abuse to addiction, causing an increase of intake and drug-seeking behaviors (Koob & Schulkin, 2019; Miczek et al., 2008; Montagud-Romero et al., 2016, 2018; Ruisoto & Contador, 2019). Social defeat (SD) is one of the most commonly used animal models to study the effects of stressful experiences. In this model, the experimental subject is repeatedly confronted with an aggressive opponent mouse (Miczek et al., 2004). SD induce a short-term increase in consumption of ethanol (EtOH) using oral self-administration (SA) with a higher motivation to get the drug (Van Erp & Miczek, 2001; Norman et al., 2015). In a previous study, we found that the effects of repeated social defeat (RSD) can be long-lasting, since mice exposed to RSD during adolescence showed higher ethanol consumption rates and a greater motivation to get the drug during adulthood (Rodríguez-Arias et al., 2016). Even after 6 months since the last stress exposure, defeated animals showed an enhanced motivation for ethanol intake (Riga et al., 2014). In addition, many studies support that social stress is one of the most important factors that influence the increase and escalation in ethanol consumption. Using voluntary ethanol intake, in the two bottle choice (TBC) task, SD produces an escalation in the consumption of alcohol after 10 days since the last exposure of stress (Norman et al., 2015; Hwa et al., 2016; Karlsson et al., 2017; Newman et al., 2018), although this effect is not observed immediately after being exposed to stress (Lopez et al., 2016). This increase in ethanol consumption induced by social stress could be due to stress-induced neuroadaptations, which ultimately produce changes in the hypothalamic, extrahypothalamic and mesocorticolimbic circuits, which are related to stress and reward (Holly et al., 2016; Hwa et al., 2016; Laine et al., 2017; Newman et al., 2018).

Nowadays, physical activity has emerged as a modulator of higher mental functions. Voluntary wheel running (VWR) in rodents produces enhanced learning, neurogenesis, angiogenesis, increases in neurotrophic factors and changes in several signaling molecules, as well as a reduction in behaviors associated with stress (Salam et al., 2009; Mul, 2018). VWR exercise after SD reduced social avoidance and anhedonia in rodents (Mul et al., 2018; Watanasriyakul et al., 2018; Zhang et al., 2019). It is known that physical exercise regulates some components of the hypothalamic-pituitary-adrenal axis (HPA), generating an adaptive response to stress (Pietrelli et al., 2018). Moreover, rats exposed to long-term access to VWR showed alterations in gene transcription factors involved in reward and dopaminergic neurotransmission in the mesolimbic reward pathway, developing conditioned place preference (CPP) to the compartment associated with physical exercise (Greenwood et al., 2011). Therefore, mice consumed significantly less ethanol in the unlimited accesTBC model when they had access to the wheel (Ehringer et al., 2009; Darlington et al., 2014, 2016).

A number of recent reports have studied the relationship between stress, addiction and the immune system. Both exposure to stress and ethanol consumption activate the immune system and induce neuroinflammation (Calcia et al., 2016; Ferrer-Pérez et al., 2018; Finnell & Wood, 2016; Rodríguez-Arias et al., 2017; Montagud-Romero et al., 2018). Moreover, deregulation in chemokine signaling and neuroinflammation have been proposed to contribute to cognitive dysfunction and mental illness (Keogh & Parker, 2011; Wohleb et al., 2013; Pascual et al., 2015). SD-induced neuroinflammation has been clearly demonstrated, characterized by an activation of microglia (Stankiewicz et al., 2015), an increase of pro-inflammatory cytokines (Ferrer-Pérez et al., 2018; Wohleb et al., 2011, 2012, 2014), or the cross of peripheral immune cells to the CNS due to higher blood-brain barrier (BBB) permeability (Rodríguez-Arias et al., 2017).

There are no current studies evaluating the role of VWR in ameliorating the increase in EtOH consumption induced by SD. In mice and humans, several studies suggest that excessive or forced physical exercise produces brain injury and neuroinflammation (Paolucci et al., 2018; Svensson et al., 2016). However, physical exercise also upregulates tight-junction associated proteins of the BBB and protects the brain from injury, reducing the activation of microglia and cytokine levels in the hippocampus in mice (Park et al., 2016; Spielman et al., 2017) and humans (Paolucci et al., 2018). Therefore, it is necessary to evaluate if the neuroinflammatory process induced by RSD mediates the increase in EtOH consumption and if VWR could modify it. The aim of the present study was, firstly, to confirm that RSD induces a long-lasting increase in EtOH consumption using oral EtOH SA when experienced during adulthood; secondly, to evaluate if VWR could decrease these RSD effects on EtOH; and finally, to evaluate the neuroinflammatory response induced by RSD and EtOH, measuring the striatal levels of two chemokines fractalkine (CX3CL1) and SDF-1 (CXCL12). Chemokines are a family of small cytokines with chemo-attraction characteristics. Social stress is known to intervene in the signaling of chemokines on microglial morpho-functional activity (Milior et al., 2016; Sawicki et al., 2015; Wohleb et al., 2013) and, in addition, the striatal levels CX3CL1 increase after EtOH intake (Pascual et al., 2015).

2. Material and methods

2.1 Subjects

A total of 115 male OF1 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21 (4 animals were discarded during the training phase of SA). All mice (except those used as aggressive opponents) were housed in groups of five in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998)

(n = 15 adult mice). All mice were housed under the following conditions: constant temperature, a reversed light schedule (lights off at 08:00 and on at 20:00), and food and water were freely available ad libitum, except during the behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (University of Valencia).

2.2 Drugs

For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was dissolved in water.

2.3 Experimental design

A first set of mice were employed to corroborate that social stress produces neuroinflammation. Animals were sacrificed 3 h after the first exploration (Control group), the first and the fourth RSD and 3 weeks after the last exposure to RSD.

Our main objective was to confirm the increase of voluntary ethanol consumption in defeated animals and to evaluate the action of physical exercise on this social stress effect. Animals were exposed to RSD (RSD and RSD + Wheel groups) or exploration condition (EXP and EXP + Wheel). Furthermore, the EXP + Wheel and RSD + Wheel groups were exposed, individually and in a different cage from the usual mice home cage, to wheel activity three days a week from PND 40 until the end of the SA procedure. This groups trained in the wheel for 1 h before each RSD or exploration condition. Three weeks after the last exposure to RSD, the animals started the EtOH SA protocol for approximately 28 days. Brain samples were also obtained 24 h after the SA procedure.

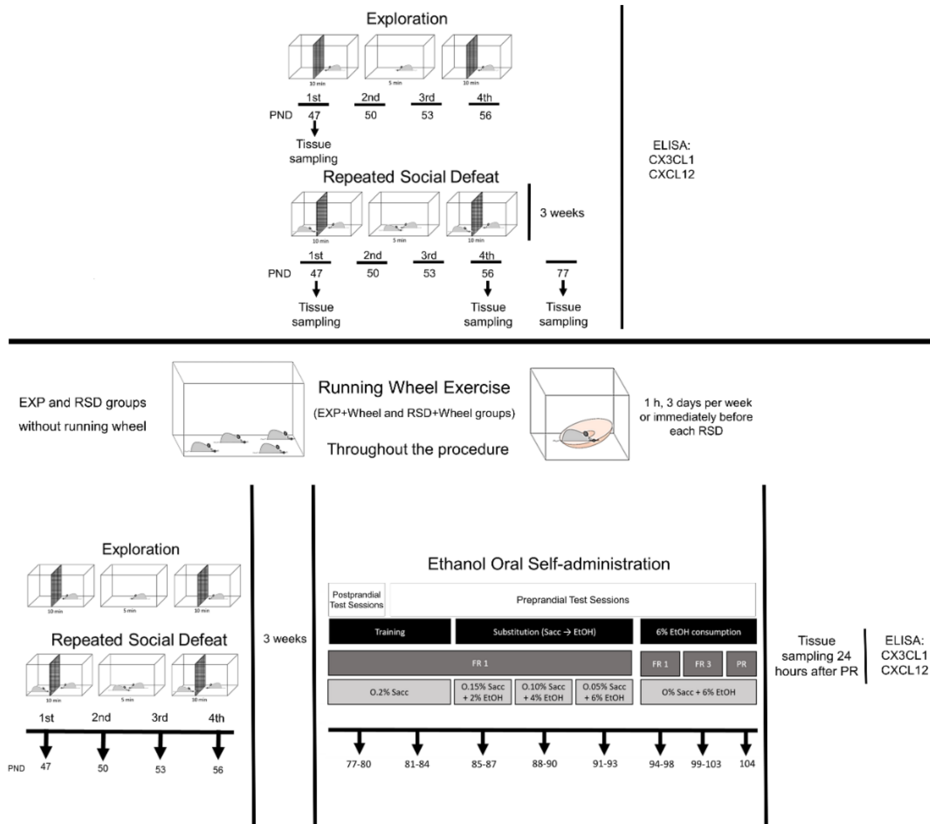

Fig. 1. Experimental design.

2.4 Repeated social defeat

Animals in the stress/defeated groups were exposed to 4 episodes of RSD lasting 25 min each on PND 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the aggressive opponent (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each RSD protocol was video-recorded and ethologically analyzed. Threat and attack behaviors were scored in aggressive

opponent mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was put back for a further 10 min to allow social threats from the aggressive opponent. The non-stressed exploration groups underwent the same protocol, but without the presence of an aggressive opponent mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, after which the behavioral tests began (see Fig. 1). In the corresponding groups, animals ran on the wheels immediately before each RSD or exploration (control group).

2.5 Apparatus and procedures

2.5.1 Oral ethanol self-administration

This procedure is based on the one employed by Navarrete et al. (2014). Oral ethanol SA was carried out in 7 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Packing software (Cibertec, SA, Spain) controlled stimuli and fluid delivery and recorded operant responses. The chambers were equipped with a chamber light, two nose-poke holes, one receptacle to deliver a liquid solution, one syringe pump, one stimulus light, and one buzzer and were placed inside noise isolation boxes. Active nose-poke delivered 36 μL of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6-s time-out period. The inactive nose-poke did not produce any consequence.

To evaluate the consequences of RSD on the acquisition of oral EtOH SA, animals underwent an experiment carried out in three phases: training, saccharin fading and 6% EtOH consumption.

Training phase (8 days): Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withheld for 24 h, and food was provided 1 h prior to the 1 h session to increase the animals' motivation. During the subsequent 3 days, water was provided *ad*

libitum, except during the 1-h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1-h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced in the mice weight loss of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 μL of 0.2 % (w/v) saccharin reinforcement.

Saccharin fading (9 days): The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% EtOH; 0.10% Sac −4% EtOH; 0.05% Sac −6% EtOH).

6% ethanol consumption (11 days): The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 6% EtOH (w/v) intake and the motivation to drink. After each session, the alcohol that remains in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and EtOH consumption (μL) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, a fixed ratio 3 (FR3) (mice had to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement, according to the aforementioned scale (for

example, if an animal activates the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40). All the sessions lasted for 1 h, except the PR session, which lasted for 2 h.

2.5.2. Low-profile running wheel

The type of wheel used was the low-profile running wheel (Med Associates Inc.), which rotates on a central axis in a horizontal plane, allowing physical activity to be carried out through natural exercise as in spontaneous locomotion. These wheels have an ideal size (10.25 × 15.5 × 13.7) to be introduced into the home cages of rodents and are linked to a monitoring system (Hub) that runs on batteries and can register the activity through a set of programs (Wheel Manager Software). All mice were housed in groups of five in plastic cages throughout the experiment. However, mice in the exercise condition (EXP + Wheel and RSD + Wheel) were individually placed in a plastic cage different to their home cage with one low-profile running wheel. In our laboratory, we have eight low-profile running wheels. All animals in the exercise condition were distributed in batches of eight to run on the wheel for 1 h, three times a week (Monday, Wednesday, and Friday) or immediately before exposure to RSD.

2.6 Tissue sampling

Striatum samples were taken 3 h after the first and the fourth RSD. Likewise, another sample was taken three weeks later and a final sample was obtained after the end of the SA procedure.

To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed, the striatum dissected following the procedure described by Heffner et al. (1980) and kept in dry ice until storage at −80 °C. Before CX3CL1 and CXCL12 determination, brains were homogenized and

prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 mL of cold lysis buffer (1% NP-40, 20 mM Tris−HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

2.7 Determination of CX3CL1 and CXCL12 levels

To determine the CX3CL1 and CXCL12 concentration on tissues, we used a Mouse CX3CL1 ELISA Kit obtained from Abcam (Ref: ab100683) and a Mouse CXCL12 Kit obtained from Abcam (Ref: ab100741) that were used following the manufacturer's instructions.

To determine the absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450 nm and final results were calculated using a standard curve following the manufacturer's instructions and expressed as ng/mg for CX3CL1 and as pg/mg for CXCL12 (tissues).

2.8 Statistical analysis

The data of the ethological analyses of opponent and intruder mice were analyzed by a two-way ANOVA with a one between-subjects variable—Exercise (with or without physical exercise)—and a one within variable—RSD encounter—with two levels: first and fourth RSD.

To analyze acquisition of EtOH SA, a three-way ANOVA was performed with a two between-subjects variable - Stress (EXP or RSD) and Exercise (with or without wheel access)—and a within-subjects variable—Days, with five levels of FR1 or FR3—followed by the Student's-Newman−Keuls test to compare the groups at

different time points of the oral self-administration paradigm. A two-way ANOVA was employed to compare the effects of RSD on the number of active responses, breaking point values and ethanol consumption during PR with two between-subjects variable—Stress (EXP or RSD) and Wheel (with or without physical exercise).

Pearson's coefficient was calculated to determine possible relationships between the EtOH consumption variable (during FR1, FR3 or PR schedules) and ethological analyses of the behaviors exhibited by the intruder mice during RSD (first and fourth).

Data related to chemokine concentrations were analyzed by a one-way ANOVA. In the first set of animals, we analyzed the effects of RSD using an ANOVA with one between-subjects variable—Stress, with four levels (Control, first RSD, fourth RSD, 3 Weeks). For the second set of animals, after SA procedure, we used a two-way ANOVA, with two between-subjects variable—Stress (EXP or RSD) and Wheel (with or without physical exercise). The ANOVAs were followed by a Bonferroni's post-hoc test. The results are reported as mean ± S.E.M. All analyses were performed using SPSS v24.

Cohen's d effect sizes were calculated for all statistically different comparisons. Effect sizes were classified as small (d = 0.20–0.49), moderate (d = 0.50–0.79), and large (d ⩾ 0.80) (Cohen, 2013).

3. Results

3.1. VWR did not affect behaviors during RSD

The ANOVA revealed a significant effect of the variable Day for Defensive/Submissive [$F(1,18) = 50.932$; $p = 0.000$], for Attack [$F(1,18) = 16.357$; $p = 0.001$], and Threat [$F(1,18) = 5.872$; $p = 0.026$] behaviors (Table 1). All mice showed an increase of the time spent in these behaviors in the last RSD compared to

the first ($p = 0.001$, $d = 2.244$; $p = 0.001$, $d = 1.290$; and $p = 0.026$, $d = 0.557$ respectively).

Table 1. Ethological analyses of the RSD.

			Without exercise		With exercise (VWR)	
		Encounters	1st	4th	1st	4th
Intruder mice	Avoidance/ Flee	Time (s)	49±6	49±9	51±7	45±6
		Latency	8±4	12±10	5±1	8±3
	Defense/ Submission	Time (s)	51±11	106±10***	44±7	105±6***
		Latency	21±13	5±2	10±2	5±2
Opponent mice	Threat	Time (s)	14±4	16±3*	10±7	13±2*
		Latency	7±3	6±2	14±11	20±11
	Attack	Time (s)	54±5	83±11***	52±5	84±9***
		Latency	6±3	2±1	3±1	3±1

Results are presented as mean values ± SEM. *$p < 0.05$; ***$p < 0.001$ differences between first and fourth RSD.

3.2. RSD increase CX3CL1 and CXCL12 levels in the Striatum

The ANOVA indicated that the exposure to RSD induced a significant increase in CX3CL1 [$F(3,28) = 3.988$; $p = 0.017$] and CXCL12 [$F(3,28) = 5.304$; $p = 0.005$] protein levels in the Striatum (Fig. 2 a and b) after the fourth RSD compared to the control group ($p = 0.019$; $d = 1.831$ for CX3CL1; $p = 0.019$; $d = 1.998$ for CXCL12).

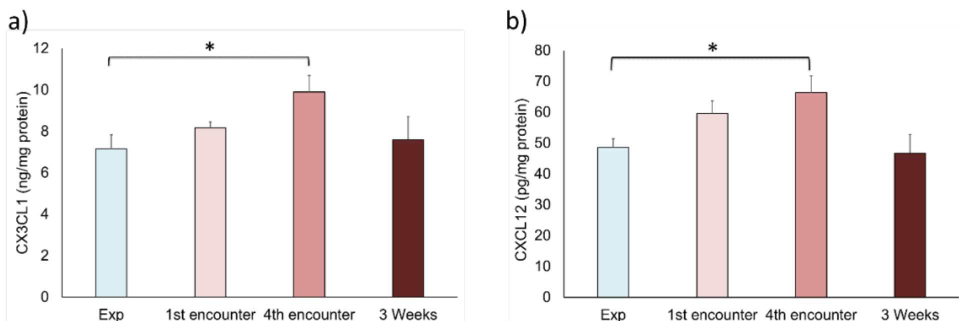

Fig. 2. RSD increase CX3CL1 and CXCL12 levels in the Striatum. (a) Concentration of CX3CL1 after exploration (EXP), first RSD, fourth RSD and 3 weeks after the last exposure to RSD. The columns represent the mean and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) of OF1 mice (n = 8 in all groups). (b) Concentration of CXCL12 after exploration (EXP), first RSD, fourth RSD and 3 weeks after the last exposure to RSD. The columns represent the mean and the vertical lines ± SEM of concentration levels of CXCL12 (pg/mg protein) of OF1 mice (n = 8 in all groups). *$p < 0.05$ with respect EXP group.

3.3. VWR counteracts the increase in ethanol oral self-administration induced by RSD

The analyses of the acquisition and substitution phases of the self-administration can be found on the supplementary data.

The ANOVA for the number of active responses during the FR1 schedule of EtOH SA revealed a significant effect of the interactions Days × Stress [$F(4,208) = 4.024$; $p = 0.032$] and Stress × Exercise [$F(1,52) = 4.959$; $p = 0.030$] (Fig. 2a). The post-hoc comparison showed that active responses were lower on day 1 compared to day 2 ($p = 0.05$; $d = 0.509$), 3 ($p = 0.001$; $d = 0.619$) and 5 ($p = 0.047$; $d = 0.356$) only in defeated animals. Defeated animals (RSD group) showed higher number of active responses than controls (EXP) ($p = 0.000$; $d = 1.31$), as well as the defeated mice exposed to wheels (RSD + Wheel) ($p = 0.000$; $d = 1.293$). With respect to EtOH consumption, the ANOVA revealed a significant effect of the interaction Stress × Exercise ([$F(1,52) = 10.124$; $p = 0.002$] (Fig. 2b). The post-hoc comparison showed that defeated animals (RSD) showed higher EtOH consumption rates than controls (EXP) ($p = 0.000$; $d = 1.566$) and the defeated mice exposed to wheels (RSD + Wheel) ($p = 0.000$; $d = 1.288$).

During the FR3 schedule, the ANOVA revealed a significant effect of the variable Days [$F(4,52) = 5.387$; $p = 0.000$] and variable Exercise [$F(1,52) = 4.959$; $p = 0.030$]

for the number of active responses (Fig. 2a). The post-hoc comparison showed a lower number of active responses on day 1 with respect to days 2 ($p = 0.003$; $d = 0.461$), 3 ($p = 0.017$; $d = 0.417$) and 4 ($p = 0.023$; $d = 0.398$). A lower number of active responses was observed in animals that had had access to wheels (EXP + Wheel and RSD + Wheel) ($p = 0.030$; $d = 0.606$). With respect to EtOH consumption, the ANOVA revealed a significant effect on the interaction Days × Exercise [$F(4,208) = 3.546$; $p = 0.008$]. The post-hoc comparison showed that animals without access to a wheel (EXP and RSD) consumed significantly more EtOH with respect to animals with VWR on day 2 ($p = 0.000$; $d = 0.916$). In addition, EXP and RSD groups also showed a significant decrease in EtOH consumption during day 1 compared to days 2 ($p = 0.000$; $d = 0.928$), 3 ($p = 0.005$; $d = 0.570$) and 4 ($p = 0.011$; $d = 0.547$) (Fig. 3b).

During the progressive ratio, for breaking point values (Fig.3c) the ANOVA revealed a significant effect of the interaction Stress × Exercise [$F(1,52) = 4.379$; $p = 0.041$]. Post-hoc comparison showed that the breaking point values were higher in defeated animals with respect to the control group ($p = 0.019$; $d = 0.940$) and RSD + Wheel ($p = 0.010$; $d = 1.068$). The ANOVA for the numbers of rewards (Fig.3e) also revealed a significant effect of the variable Exercise [$F(1,52) = 8.281$ ($p = 0.006$), since the groups exposed to VWR showed a lower number of rewards ($p = 0.006$; $d = 0.773$). No effects were observed for EtOH consumption (Fig.3d).

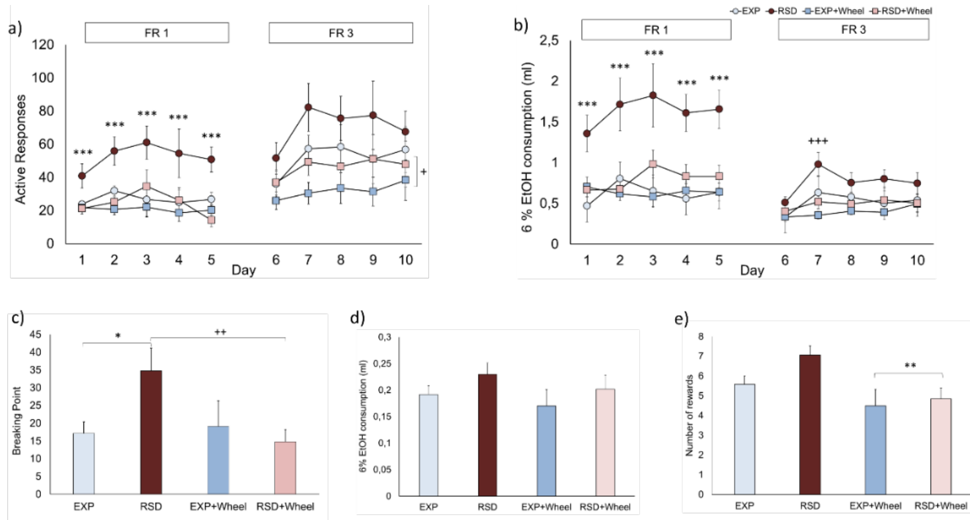

Fig. 3. Effects of running wheel on the increase in oral EtOH self-administration induced by RSD in OF1 mice. Animals were divided into the following four treatment groups: EXP group allowed to explore a new cage and without access to a running wheel (EXP, n = 14) or EXP group allowed to explore a new cage and with access to a running wheel (EXP + Wheel, n = 14); and RSD group exposed to RSD and without access to a running wheel (RSD, n = 15) or RSD group exposed to RSD and with access to a running wheel (RSD + Wheel, n = 13). The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% EtOH consumption and (e) the number of rewards obtained during PR. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ significant difference with respect to the control and RSD + Wheel groups; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ significant difference between groups with running wheel access vs. groups without access.

3.4. Passive coping during RSD correlated with stronger ethanol consumption during FR1 schedule.

Pearson's coefficient showed a positive correlation between the time spent in Avoidance/Flee behaviors during the 4th RSD and the average consumption of ethanol during FR1 schedule ($r = 0.699$; $p = 0.024$) in RSD group (Fig.4). Those mice that spent more time in avoidance/flee behaviors during RSD showed higher consumption rates of EtOH during the FR1 schedule.

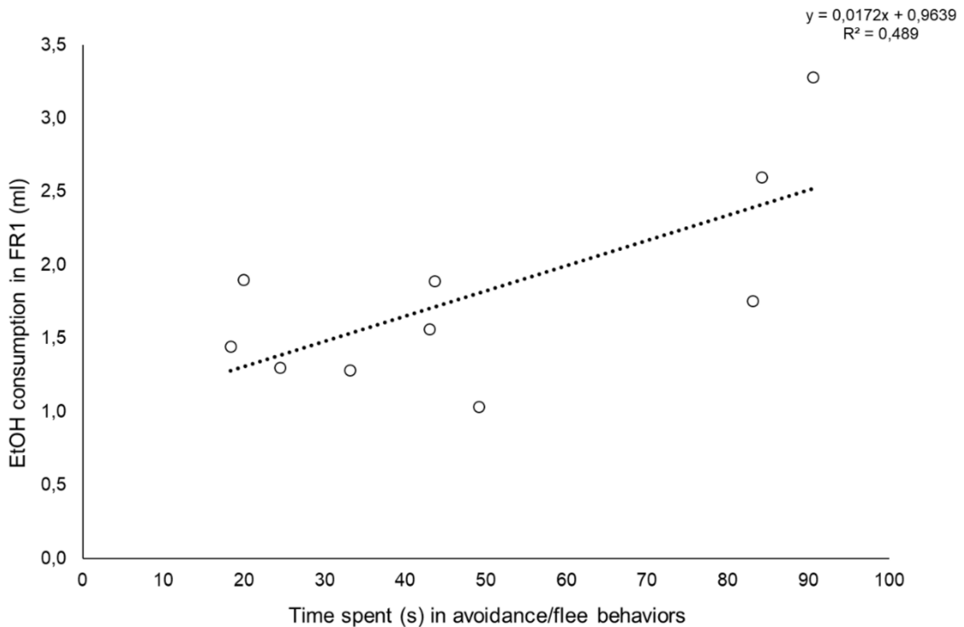

Fig. 4. Regression plot for the Pearson correlation between Avoidance/Flee behaviors (fourth RSD) and the average consumption of ethanol during FR1 schedule. The trend line represents the linear regression of data ($y = 0.017x+0,9639$; $r2 = 0.489$).

3.5. VWR reverses the increase in striatal levels of CX3CL1 and CXCL12 induced by RSD after EtOH SA

The ANOVA revealed a significant effect of the interaction Stress × Exercise [F (1,27) = 8.948; p = 0.006] on CX3CL1 protein levels after oral SA of EtOH (Fig. 5a). The post-hoc comparison revealed that defeated animals (RSD) presented significantly higher levels of CX3CL1 than non-stressed animals (EXP) (p = 0.009; d = 2.550). In addition, defeated animals exposed to physical exercise (RSD + Wheel) showed significantly lower protein levels compared to defeated animals without access to a running wheel (RSD) (p = 0.000; d = 4.803).

Regarding CXCL12 protein levels, the ANOVA revealed a significant effect of the interaction Stress × Exercise [F (1,27) = 4.849; p = 0.036] after oral SA of EtOH (Fig. 5b). The post-hoc comparison revealed that the defeated group (RSD) showed significantly higher protein levels than the non-defeated group (EXP) (p = 0.001; d = 2.213). Higher levels of CXCL12 were obtained in the defeated group without access to the running wheel (RSD) compared to defeated group with VWR (RSD + Wheel) (p = 0.002; d = 2.163).

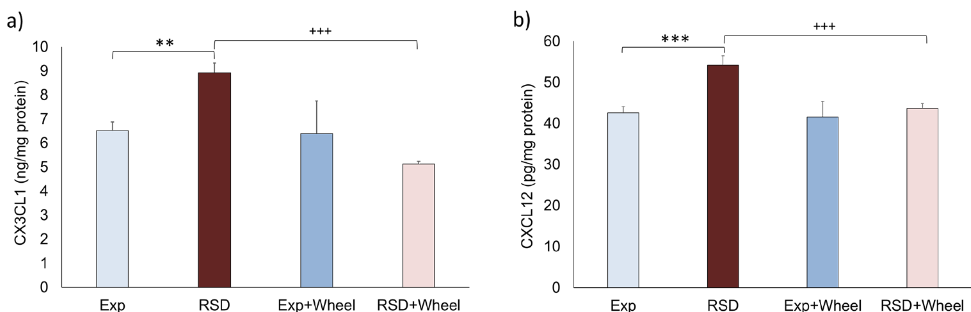

Fig. 5. VWR reverses the increase in striatal levels of CX3CL1 and CXCL12 induced by RSD after EtOH SA. (a) CX3CL1 protein levels and (b) CXCL12 protein levels after oral EtOH SA in the following four treatment groups: EXP group allowed to explore a new cage and without access to the running wheel (EXP, n = 8) or EXP group allowed to explore

a new cage and with access to the running wheel (EXP + Wheel, n = 7); and RSD group exposed to RSD and without access to the running wheel (RSD, n = 8) or RSD group exposed to RSD and with access to the running wheel (RSD + Wheel, n = 8). The columns represent the mean and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice. **p < 0.01, ***p < 0.001 significant difference with respect to the EXP group; +++p < 0.001 significant difference with respect to the corresponding EXP or RSD groups.

4. Discussion

The present study confirmed that social stress experienced during adulthood increases consumption and motivation for ethanol and that VWR reverted this effect. In addition, we corroborated that RSD produces a neuroinflammatory response by increasing protein levels of the chemokines CX3CL1 and CXCL12. VWR was also able to revert the neuroinflammatory response caused by stress and exposure to ethanol.

In a previous study from our laboratory, we observed that mice subjected to social stress during adolescence showed an increase in consumption and motivation for ethanol in the oral SA paradigm (Rodríguez-Arias et al., 2017). Those results agree with the results obtained in this study in mice defeated during adulthood. The effect of social stress was more apparent during the FR1 phase, although increases were also observed during FR3. The greater the number of active responses needed, the less value the reward has, since there is a loss in the value of delayed rewards (Lagorio & Winger, 2014; Mazur, 1986). During the first day of FR3 a decrease in active responses is characteristically observed because the animals need to learn the new demands. Control non-stressed groups tend to decrease the interest in getting infusions, perhaps because their seeking behavior is not strong (Samson & Czachowski, 2003), although stressed animals work harder to obtain EtOH. Therefore, during the FR3 schedule, it is normal to observe a decrease in the number of infusions received and the total consumption of EtOH with respect to FR1, while

defeated animals maintain a greater consumption. Equally, the PR schedule determines the breaking point or limit of active responses that the animal is willing to carry out to obtain EtOH. This progressive pattern of responses is usually indicative of seeking behavior related to motivation (Samson & Czachowski, 2003). We observed that defeated animals showed higher breaking points, although no more EtOH consumption was observed. Taking into account the progressive nature of PR schedules, a small number of substance intakes is usually observed due to the limited session time (in our case, 2 h) (Bickel et al., 1990). For this reason, it is difficult to observe differences in consumption. The PR schedule is complementary to the FR schedules, as it links the seeking, motivation, and maintenance behaviors of addictive behavior. In agreement with our results, other studies showed that rodents exposed to SD during adolescence (Marcolin et al., 2019) or adulthood (Deal et al., 2018; Lopez et al., 2016; Riga et al., 2018) showed an increase and escalation in voluntary consumption of ethanol.

Recent studies suggest that coping strategies are associated with resilience or vulnerability to stress (Chen et al., 2015; Finnell et al., 2017; Pearson-Leary et al., 2017; Wood et al., 2015), which seems to be related with neurochemical adaptations that specifically affect the function of the dopamine system and could therefore modify the rewarding efficacy of drugs of abuse (Brodnik et al., 2017). In the present study, we have observed that those animals that spent more time in avoidance and flee behaviors during the last SD presented higher EtOH intake. We have obtained similar results in a previous study (Ródenas-González et al., 2020), where we observed a positive correlation between flight and avoidance behaviors and the increase in the conditioned rewarding effects of cocaine in the CPP. These results confirm that active coping and adequate adaptation to stress reduces the increase in the rewarding effects of drugs of abuse induced by social stress. Other studies have also confirmed that mice showing active coping strategies show less anhedonia (Wood et al., 2015), less anxiety and greater social interaction (Duclot et al., 2011;

Hollis et al., 2011; Kumar et al., 2014). In this work, we hypothesized that controlled physical activity could reduce the effects of RSD. VWR is a rodent model that mimics several aspects of human physical exercise training (Mul et al., 2018). There are no previous studies where VWR was used to intervene on ethanol consumption and motivation induced by social stress, but the effect of physical exercise on ethanol consumption has been studied in various paradigms of voluntary consumption of ethanol, such as TBC or the free-choice paradigm. In general, these studies show lower consumption rates of alcohol in male (Ehringer et al., 2009; Hammer et al., 2010) and female rodents (Piza-Palma et al., 2014) with access to the running wheel. However, a recent study pointed out that the removal of access to exercise appeared to enhance ethanol intake/preference (Lynch et al., 2019).

On the other hand, physical exercise has a well-documented beneficial effect on stress-related mental disorders. VWR counteracted the development of social avoidance and anhedonia after chronic SD stress (Mul et al., 2018; Zhang et al., 2019). Likewise, VWR attenuated the increased neuroendocrine response induced by social isolation stress (Watanasriyakul et al., 2019) and counteracted the behavioral impairments induced by uncontrollable stress (Greenwood et al., 2003, 2012, 2013; Tanner et al., 2019). The relationship between stress and exercise is bidirectional, as Parra-Montes de Oca et al. (2019) have recently reported that chronic stress decreases the metabolic response to voluntary exercise characterized by the loss of white adipose tissue depots.

Our study showed that exposure to VWR was capable of decreasing the long-lasting increase in ethanol intake induced by RSD. This counteracting action seems to be specific to the stress-induced effect, as no differences in ethanol intake were observed between the two non-stressed groups, meaning that exposure to VWR did not affect basal ethanol intake. In addition, the ethological analyses of RSD showed no differences in opponent or intruder mice behaviors depending on the exposure to

VWR, meaning that previous exposure to exercise before each RSD did not affect social stress.

Inflammatory stimuli induced the release of inflammatory cytokines as well as chemokines that functioned as chemo-attractants, presenting homeostatic and/or inflammatory functions (Koper et al., 2018). SD have been linked to an increase of the neuroimmune response, including the activation of microglia (Wohleb et al., 2011, 2014; Lehmann et al., 2016; Rodríguez-Arias et al., 2018), the increase in BBB permeability (Rodríguez-Arias et al., 2016), and the increase of IL-6 levels in plasma and the striatum (Ferrer-Pérez et al., 2018). We have now corroborated these results, showing that RSD also induced an increase in the chemokines CX3CL1 and CXCL12 after the fourth RSD.

There is no consensus whether CX3CL1 is an inflammatory or a pro-inflammatory chemokine (Mecca et al., 2018; Rahman et al., 2011). CX3CL1 signaling through its receptor Cx3cr1 which is only expressed in microglia, being critical for the microglia-neuron cross-talk (Jones et al., 2010; Lauro et al., 2015; Poniatowski et al., 2017). The stress-induced changes in CX3CL1 are not clear, as discrepant results have been described. Adult male rats exposed to chronic mild stress for 2 weeks not only showed an increase in CX3CL1 expression in the dorsal hippocampus, but also a decreased expression in the prefrontal cortex (Rossetti et al., 2016). Moreover, the same authors observed increases or decreases in CX3CL1 expression in the hippocampus after seven weeks of chronic mild stress. Although we have reported in the present study an increase of striatal CX3CL1 levels after the fourth RSD, we obtained in a recent report the opposite effect, following the same experimental procedure with a decrease in CX3CL1 levels in the striatum and no changes in the hippocampus (Montagud-Romero et al., 2020). The use of a different strain of mice (OF1 or C57BL/6NTac) could be responsible for these discrepant results. Since OF1 is a particularly territorial strain of mice, the loss of social encounters could have had

a more intense stress effect leading to a higher neuroinflammation response. In addition, in the present study, we observed an increase in CX3CL1 levels in defeated mice after ethanol oral self-administration.

With respect to CXCL12, this chemokine is ubiquitously expressed and binding to two receptors, CXCR4 and ACKR3. The CXCL12/CXCR4/ACKR3 axis plays key roles in many physiological and pathological processes, including embryogenesis, wound healing processes, angiogenesis, homeostasis and it also participates in the progression of inflammation (García-Cuesta et al., 2019; McCandless et al., 2006; Niraula et al., 2018). Therefore, an increased expression of CXCL12 has been described in many inflammatory and autoimmune diseases (Rizzo et al., 2013; Wei et al., 2012), which suggests an inflammatory role. In agreement with this role, we observed that our RSD protocol induced a significant increase of this chemokine after the fourth defeat. However, Sawicki et al. (2015) did not observe changes in the CXCL12 gen-expression or a reduction of CXCL12 mRNA levels in enriched microglia/ macrophages immediately after the last exposure to SD.

Many studies show that a prolonged consumption of ethanol produces increases in brain chemokine levels in rodents (Pascual et al., 2015; Somkuwar et al., 2016). In our study, both chemokines were significantly increased after oral ethanol SA in defeated animals in comparison with those non-stressed.

The increase in chemokines after EtOH oral SA, which was only found in the defeated group that had no access to VWR, suggests a sensitization of the neuroinflammatory response. Stressed mice exposed to ethanol presented higher levels of chemokines that are not observed in non-stressed animal or in those defeated but exposed to VWR. These differences could be due to the less amount of ethanol ingested by these mice, since it is well known that ethanol is per se a potent neuroinflammatory factor (Montesinos et al., 2016; Pascual et al., 2015). However, we have previously reported that defeated mice showed elevated levels of the pro-

inflammatory cytokine IL-6 that were not observed in control mice after having been exposed to the same doses of cocaine (Ferrer-Pérez et al., 2019). Therefore, although we cannot prevent control mice to ingest less ethanol, we suggest that social stress sensitized the inflammatory system to further responses. Confirming these results, we have previously reported increases in these chemokines after cocaine administration in mice (Araos et al., 2015), In addition, we observed a decrease in CXCL12 chemokine in abstinent cocaine users without changes in CX3CL1 plasma levels, although these levels positively correlated with the cocaine symptom severity for cocaine abuse/dependence (Araos et al., 2015). Although we did not measure the acute effect of VWR after each RSD, we observed that after oral EtOH SA, VWR significantly decreased the striatal levels of chemokines (CX3CL1 and CXCL12), showing levels similar to those in the control group. In contrast with this effect, there are several reports showing an increased neuroinflammatory response in animals exposed to physical exercise, in some cases forced or maintained for 24 h a day (Pinto et al., 2019; Svensson et al., 2016). The literature suggests that moderate-intensity exercise may be optimal in decreasing neuroinflammatory markers (Henrique et al., 2018; Paolucci et al., 2018). In agreement with our results, some studies have shown the positive effects of controlled physical exercise on stress (Ignácio et al., 2019; Mul et al., 2018), addiction (Somkuwar et al., 2016) or Alzheimer's disease (He et al., 2017; Jensen et al., 2019; Małkiewicz et al., 2019). Physical exercise interacts with stress and neuroinflammation depending on the intensity. Several studies have observed that VWR reduces the levels of corticosterone and glucocorticoid receptors, attenuating the negative effects of chronic stress (Ignácio et al., 2019; Lynch et al., 2019; Watanasriyakul et al., 2019; Zheng et al., 2006). The inhibition of the excess production of corticosterone can attenuate the inflammatory response of stress (Niraula et al., 2018). Only few studies have examined the effect of exercise and chemokine levels. For example, long-term

wheel performance decreases the western diet increased in the gene expression of CXCL10 and CCL2 (Carlin et al., 2016).

5. Conclusions

In conclusion, our results suggest that VWR is a beneficial environmental intervention that is capable of blocking the increased ethanol intake and the neuroinflammation induced by social stress. Our work highlights the complexity of the brain mechanisms involved in the inflammatory process in response to social stress. To sum up, VWR could be a promising preventive and therapeutic target to avoid and reduce the detrimental effects induced by social stress.

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Study 3

Ethanol intake in male mice exposed to social defeat: Environmental enrichment potentiates resilience

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Abstract

Large preclinical evidence shows that exposure to social defeat (SD) increases vulnerability to drug abuse, increasing the consumption of ethanol. However, not all subjects are equally affected by the changes induced by stress. Previous reports have evidenced that the resilient phenotype to depressive-like behaviors after SD is associated with the resistant phenotype to cocaine-increased rewarding effects and the smaller neuroinflammatory response. The aim of the present study was to further clarify whether the resilient profile to depressive-like behavior also predicts a protection against the increase in ethanol intake induced by SD. The neuroinflammatory profile was studied after the end of the oral ethanol self-administration (SA) procedure, measuring levels of the pro-inflammatory cytokine IL-6 and the chemokine CX3CL1 or fractalkine in the striatum and prefrontal cortex. Previous studies have shown that environmental enrichment (EE) is an effective mechanism to dimish the detrimental effects of social stress. In a second study, we aimed to evaluate if EE housing before exposure to SD could potentiate resilience. Our results showed that mice with a phenotype susceptible to SD-induced depressive-like behaviors showed increased ethanol consumption and increased neuroinflammatory signaling. In contrast, despite the lack of effect on depressive-like behaviors, defeated mice previously housed under EE conditions did not show an increase in ethanol SA or an increase in immune response. To sum up, the resilient phenotype to SD develops at different levels, such as depressive-like behaviors, ethanol consumption and the neuroinflammatory response. Our results also point to the protective role of EE in potentiating resilience to SD effects.

Keywords: Neuroinflammation; Social defeat; Ethanol; Susceptibility; Resilience; Environmental enrichment

Abbreviations:

AUD: alcohol use disorder; BDNF: brain-derived neurotrophic factor; CPP: conditioned place preference; CRF: corticotrophin-releasing factor; CX3CL1: C-X3-C motif ligand 1 (fractalkine); EE: environmental enrichment; ELISA: enzyme-linked immunosorbent assay; FR1: fixed ratio 1; FR3: fixed ratio 3; HPA: hypothalamic-pituitary-adrenal; IL: infralimbic cortex; IL-6: interleukin 6; NAc: nucleus accumbens; NLRP3: Nod-like receptor pyrin containing 3; PFC: prefrontal cortex; PND: postnatal day; PR: progressive ratio; PrL: prelimbic cortex; SA: self-administration; SD: social defeat; SWR: social withdrawal ratio; TLR-4: Toll-like receptor 4

1. Introduction

We are continuously exposed to different types of stress throughout our life, and stress produced by social interaction is the most common type of stress in human beings (Montagud-Romero et al., 2018). Numerous studies have shown that exposure to social stress is associated with an increase in drug use, such as cocaine (Ferrer-Pérez et al., 2018; Reguilón et al., 2017; Rodríguez-Arias et al., 2016, 2017), MDMA (García-Pardo et al., 2015) or alcohol (Beutel et al., 2018; Hwa et al., 2016; Montagud-Romero et al., 2021; Newman et al., 2018a; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Regarding alcohol, the studies to date show that both exposure to stress and the way to cope with it should be considered as predictors of alcohol consumption in humans (see review by Newman et al., 2018b). People who consume alcohol in a negative context, for example to reduce anxiety or stress, are more likely to develop a long-term problematic use and develop a chronic alcohol consumption with the corresponding negative consequences that characterize long-term alcohol abuse (e.g. negative social, physical and mental consequences; Newman et al., 2018a; Sinha, 2001). Moreover, exposure to social stress can further increase the likelihood of developing uncontrolled alcohol use or relapse (Adinoff et al., 2017).

The social defeat (SD) model is the most widely used model to study the effects of social stress (Hammels et al., 2015). SD consists of an agonistic encounter between conspecifics of the same species (Miczek et al., 2004), imitating the subordination status of human relationships (Selten et al., 2013). Exposure to SD stress induces profound physiological changes and endocrine responses, yielding a significant increase in corticosterone levels (Montagud-Romero et al., 2015; Rodríguez-Arias et al., 2017). In addition, it produces modifications in numerous neurotransmitter systems such as the serotonergic, dopaminergic or the GABAergic systems (Montagud-Romero et al., 2018).

Using this procedure, we have previously shown that exposure to four SD episodes either during adolescence or adulthood induced a long-lasting increase of ethanol self-administration (SA) during adulthood (Montagud-Romero et al., 2021; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Adolescent or adult mice were exposed to four SD episodes on alternating days and, three weeks after the last encounter, we measured oral ethanol SA. Defeated mice showed a delayed increase in ethanol consumption, made more active responses and showed increased motivation for alcohol in the progressive ratio (PR). Our results and other similar results obtained with voluntary ethanol drinking (Hwa et al., 2016; Norman et al., 2015) or indicating increased sensitivity to ethanol –induced conditioned place preference (CPP; Macedo et al., 2018)– confirmed that social stress increases vulnerability to the rewarding effects of alcohol.

Besides increasing alcohol intake, animals exposed to SD also exhibit increased anxiety and depressive-like behaviors, such as social avoidance (Blanco-Gandía et al., 2019; Ferrer-Pérez et al., 2019; Patel et al., 2019; Spijker et al., 2020). In the last decade, numerous studies have observed that the behavioral and psychological reactions to SD are not equal. Some animals are more susceptible and develop unhealthy responses, such as increased drug intake, anxiety or depressive-like behaviors. However, other subjects show resilience to stress and present a more adjusted psychological functioning (Brockhurst et al., 2015; Charney, 2004; Dantzer et al., 2018; Krishnan et al., 2007; Nasca et al., 2019).

Numerous studies have shown that passive, rather than active, coping strategies during SD are linked to stress-induced maladaptive behaviors (Ballestín et al., 2021; Hawley et al., 2010; Russo et al., 2012; Wood & Bhatnagar, 2015). Animals that display passive coping mechanisms seem to be susceptible to physiological effects and psychopathology (Hawley et al., 2010; Russo et al., 2012; Wood & Bhatnagar, 2015). However, the stress response does not only involve the coping strategies

during stress, but also its physiological processes (Murrough & Russo, 2019). The link between individual differences and the immune system response to stress is now a critical field of research (Ballestín et al., 2021; Hodes et al., 2014; Westfall et al., 2021; Wood et al., 2015). As a general result, these pre-clinical studies report lower immune system responses to depressive-like behaviors induced by social stress in resilient mice, compared to susceptible animals that developed anhedonia or social withdrawal behavior. Moreover, social stressors can modify the brain's reward system function due to the close association between the brain systems that regulate stress and the systems responsible for responses to drugs of abuse (Rodríguez-Arias et al., 2013). In contrast to the numerous reports that focus on predicting the individual response to depression-like stress consequences, only a recent study focused on the resilience and susceptibility to stress-induced enhancement of the cocaine response. We showed that resilient mice to depressive-like behaviors are also resilient to the increased cocaine reward induced by SD and exhibit a less intense neuroinflammatory response (Ballestín et al., 2021).

Although the increase in psychostimulant effects induced by SD has been thoroughly studied in the literature, there are fewer studies regarding the increased ethanol intake, and to our knowledge, only one study has focused on the resilient response to SD. Riga et al. (2020) suggest that resilience to depressive-like behaviors could protect from the development of alcohol use disorder (AUD)-like phenotypes. In their study, rats classified as depression-prone were more vulnerable to alcohol, emulating patterns of alcohol dependence as those seen in individuals with an alcohol use disorder. In this study, animals were exposed to repeated SD, and subsequently isolated for several weeks. Their depression profile was evaluated during isolation, weeks after the last defeat. In addition to social avoidance, cognitive performance was also used to further classify animals into resilient or susceptible to depressive-like behaviors. Although the authors claimed that depression-prone animals showed a more intense pattern of alcohol consumption, their increase in

alcohol intake during SA acquisition was not significantly higher. However, they observed a greater response to alcohol reward during the fixed ratio 3 (FR3) and PR schedule. In addition to high motivation toward alcohol, these depression-prone rats showed a tendency toward extinction resistance and relapse facilitation.

The present study was designed to further clarify if the resilient profile to depressive-like behavior also predicts a protection against the increase in ethanol intake induced by SD. The neuroinflammatory profile of resilient and susceptible mice were also studied after the end of the oral SA procedure, measuring levels of the pro-inflammatory cytokine interleukin 6 (IL-6) and the chemokine C-X3-C motif ligand 1 (CX3CL1) or fractalkine in the striatum and the prefrontal cortex (PFC). Both neuroinflammatory markers were reported to be differentially affected by social stress experiences in resilient or susceptible animals (Ballestín et al., 2021; Reguilón et al., 2020, 2021). To further characterize the potentiation of the resilience response, a second experiment took place to evaluate the effect of environmental enrichment (EE) exposure during adolescence, prior to the SD stress. The EE model selected for this work can be considered a basic and modest EE model, which based on the results obtained previously (Giménez-Gómez et al., 2021), we hypothesize is sufficient to stimulate resilience and block the increase in the reinforcing effects of ethanol and the neuroinflammatory response induced by SD.

2. Methodology

2.1. Animals

A total number of 87 adult male C57BL/6 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. Experimental mice were housed in groups of four in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N = 20) and were individually housed in plastic cages (21 × 32 × 20 cm) for at least one month prior to initiation of the experiments in order to

heighten aggression (Rodríguez-Arias et al., 1998). All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (red light from 8:00 to 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (number 2017-VSC-PEA-00224, on December 11th, 2017).

2.2. Drugs

For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was dissolved in water.

During the SA training phase, a 0.2% (w/v) saccharin solution in water was used. During the SA substitution phases, a mixture of 0.15% saccharin concentration dissolved in water and 2% ethanol was used for the first subphase; in the second subphase, a mixture of 0.10% saccharin solution in water and 4% ethanol was used; and, in the third subphase, a mixture of 0.05% saccharin solution in water and 6% ethanol was used.

2.3. Experimental design

The study consisted of two experiments. The experimental design between the two experiments differs only in the housing condition of the animals. In the first experiment, all the animals were housed in regular condition throughout the study. In the second experiment, all mice were housed in a consistent EE in big cages (59 x 38 × 20 cm) with PVC items such as plastic houses and tubes from PND 21 to 47. The day before the beginning of SD, mice housed in EE were moved to standard

housing conditions until the end of the SA procedure, i.e., the animals were only exposed to EE from the onset of adolescence until early adulthood or late adolescence.

All mice were exposed to the SD procedure or exploration from PND 47 to 56 (i.e., during early adulthood or late adolescence). 24 h after the last SD episode, animals performed the Test for Social Interaction to evaluate depressive-like behaviors and were characterized as resilient or susceptible depending on their social withdrawal ratio (SWR). Subsequently, three weeks after the last defeat, the animals initiated the ethanol SA protocol for approximately 28 days. At the end of this test, all the animals were sacrificed to obtain the PFC and striatum for further analysis of the cytokine and chemokine levels.

The experimental design is depicted in Figure 1.

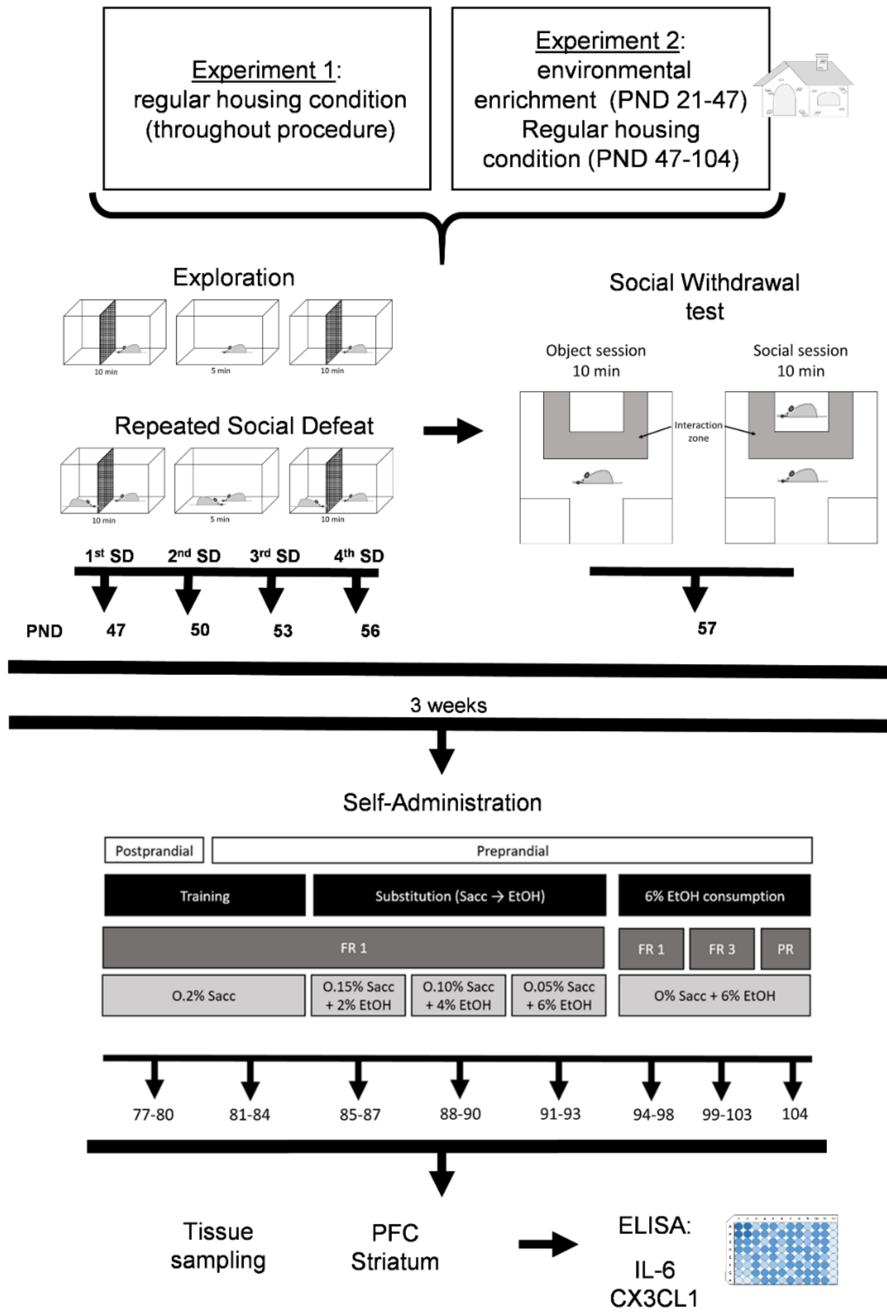

Fig. 1. Experimental design.

2.4. Procedure and apparatus

2.4.1. Housing conditions

Male mice in the regular housing condition were housed in groups of four in transparent plastic cages (27 × 27 × 14 cm) with no more enrichment than standard bedding (wood flakes 1–3.35 mm), nesting material (paper strands) and two wooden gnaw sticks (5 x 1 × 1 cm) per cage. Male mice in EE conditions were housed in groups of four in plastic cages (59 x 38 × 20 cm) with standard bedding and nesting material, two wooden gnaw sticks plus additional PVC tunnel (13 × 5.5 cm) and a plastic mouse house (12.5 x 10.5 × 11 cm; Ferrer-Pérez, 2019; Giménez-Gómez et al., 2021).

2.4.2. Procedure of social defeat (SD)

Animals in the stress/defeated groups were exposed to 4 episodes of SD during adulthood, each lasting 25 min and consisting of three phases. The initial phase began by introducing the "intruder" (the experimental animal) into the home cage of the "resident" (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-min period. Finally, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al.,

1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al. (1998).

2.4.3. Social withdrawal ratio (SWR)

The SWR used was based on the social approach-avoidance test previously described by Berton et al. (2006). The test took place 24 h after the last SD during dark cycle and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Animals were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Although it can be argued that the probe mouse used in the social interaction test resembles the aggressor, and that this could foster social aversion, this is unlikely, since previous experiments demonstrate similar amounts of social investigation, irrespective of the strain used (i.e., C57BL/6; Berton et al., 2006). Before each session, the arena was cleaned with 5% alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Locomotion and arena occupancy during object and social sessions were determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage.

Corners were defined as two squares of similar areas on the opposite wall of the arena.

2.4.4. Apparatus and procedures: Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 36 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% ethanol consumption.

2.4.4.1. Training phase (8 days)

Two days before the initiation of the experiment, access to the standard diet was restricted to 1h per day. Before the first training session, water was withdrawn for 24h, and food allotment was provided 1h prior to the session to increase the motivation for active nose-poking. During the subsequent three days, water was provided ad libitum, except during the 1h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1h after the end of each daily session and water was available ad libitum to avoid ethanol consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding

weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 µl of 0.2% (w/v) saccharin reinforcement.

2.4.4.2. Saccharin substitution (9 days)

The saccharin concentration was gradually decreased as the ethanol concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% ethanol; 0.10% Sac −4% ethanol; 0.05% Sac −6% ethanol).

2.4.4.3. 6% ethanol consumption (11 days)

The aim of the last phase was to evaluate the number of active nose-poke responses, the 6% ethanol (w/v) intake and the motivation to drink. This phase began 38 days after the last SD. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, FR3 (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a PR session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40. All the sessions lasted 1 h, except the PR session, which lasted 2 h (Navarrete et al., 2012, 2014).

2.4.5. Immunoassay analysis (ELISA)

Samples from the striatum and the PFC were obtained 24 h after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Paxinos & Franklin, 2001), which were then kept in dry ice until storage at −80 °C. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). We determined CX3CL1 and IL-6 concentration in the striatum and PFC. All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the final results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

2.5. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SWR. SWR is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups. To study the relationship between the percentages of susceptible mice in non-enriched and enriched mice, the chi-square ($\chi 2$) test was used to evaluate the categorical variables Stress and Housing.

To analyze acquisition of ethanol SA, a two-way ANOVA was performed with one between-subjects variable –Stress with three levels (Control, Resilient and Susceptible; or EE-Control, EE-SD-R and EE-SD-S)– and a within-subjects variable –Days, with five levels of FR1 or FR3–. The effects of SD and treatment on breaking point values and ethanol consumption during PR was analyzed by a two-way ANOVA, with one between-subjects variable –Stress.

The data of the CX3CL1 and IL-6 levels were analyzed using a one-way ANOVA with one between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible; or EE-Control, EE-SD-R and EE-SD-S).

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics v.26. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

In order to evaluate the differences induced by housing conditions (standard housing and environmental enrichment), we additionally performed a statistical analysis with the variable Housing for the 6 groups. For ethanol SA, we performed a two-way ANOVA with two between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible) and Housing, with two levels (SH and EE)– and for FR1 and FR3, a within-subjects variable –Days, with five levels–. The effects of SD and housing on the breaking point values and the ethanol consumption during PR, as well as the results of striatum protein levels of IL-6 and CX3CL1, were analyzed by a two-way ANOVA, with two between-subjects variables –Stress and Housing.

3. Results

3.1. Resilience to SD under regular housing conditions

3.1.1. Classification between susceptible and resilient mice according to their social withdrawal ratio.

Following the SWR calculation criteria, the control group (n = 12) showed a mean SWR higher than 1.

In the defeated group of animals (n = 30), 53.3% of the mice showed a SWR under 1, which classifies them as susceptible mice (n = 14), and the remaining 46.6% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient mice (n = 16).

3.1.2. Susceptible mice showed higher ethanol intake than resilient animals

No differences were found between the animals during training or substitution phases, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Days [$F(1,39) = 17.697$; $p < 0.001$] and Stress [$F(2,39 = 4.854$; $p < 0.01$] (Fig. 2a). The post-hoc comparison showed

that mice performed fewer active responses on days 1 and 2 compared to the last day ($p < 0.001$ in all cases). Moreover, susceptible mice performed fewer active responses than the control group ($p < 0.01$).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Stress [$F(2,35) = 4.650$; $p = 0.01$) (Fig. 2b). The post-hoc comparison showed that the susceptible group consumed ethanol at higher rates than the control (p's < 0.05) and resilient groups ($p < 0.01$).

During the FR3 schedule, the ANOVA revealed a significant effect of the interaction Days × Stress [$F(4,184) = 4.940$; $p = 0.001$) for the number of active responses (Fig. 2a). Susceptible mice showed a higher number of active responses than controls on day 10. Moreover, control animals showed a lower number of active responses on day 10 compared to day 6 ($p < 0.05$). However, susceptible mice increased the number of active responses on days 6 and 8 compared to day 10 ($p < 0.05$ and $p < 0.01$, respectively).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Days [$F(4,156) = 5.216$; $p < 0.001$], Stress [$F(2,39) = 3.949$; $p < 0.001$] and the interaction Days × Stress [$F(8,156) = 3.691$; $p < 0.001$] (Fig. 2b). Susceptible mice consumed significantly more ethanol than controls on days 7–10 ($p < 0.001$ in all cases) and than resilient mice on days 6 ($p < 0.05$), 9 ($p < 0.05$) and 10 ($p < 0.001$). Moreover, control animals consumed more ethanol on day 6 compared to the rest of the days ($p < 0.001$ in all cases). Susceptible mice also consumed more ethanol on day 10 compared to the previous days ($p < 0.01$ with respect to days 6, 7 and 9; $p < 0,05$ with respect to day 8).

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the variable Stress [$F(2,39) = 6.418$; $p < 0.004$] (Fig. 2c). The post-hoc comparison showed that the breaking point values were higher in susceptible mice with respect to control and resilient animals ($p < 0.01$ in both cases).

The ANOVA for ethanol consumption during PR did not reveal a significant effect of the variable Stress (Fig. 2d).

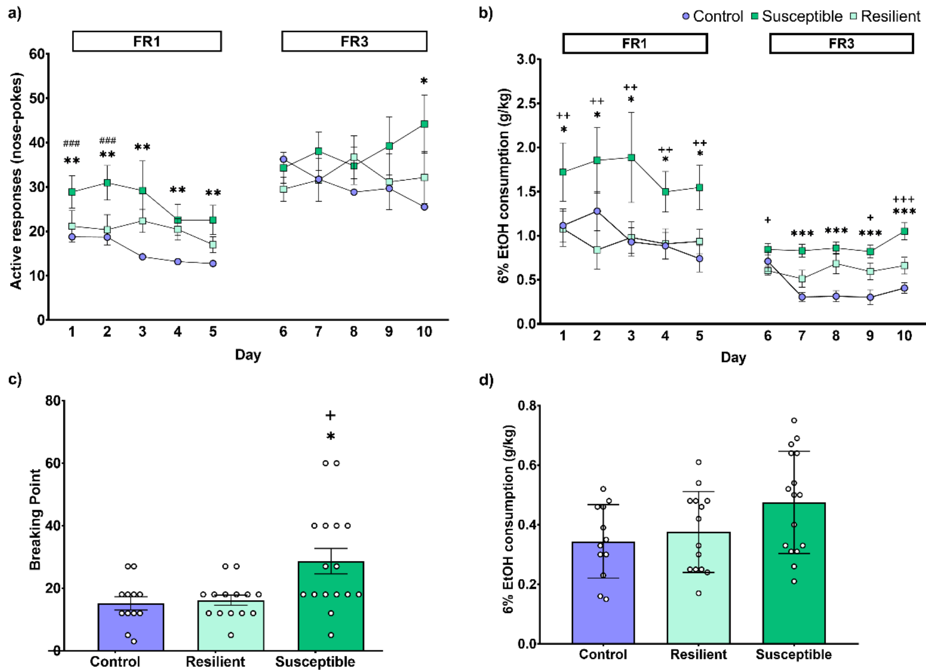

Fig. 2. Resilient mice showed lower ethanol intake than susceptible animals. Mice were divided into Control (n = 12); Resilient (n = 14) and Susceptible (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% ethanol consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% ethanol consumption during PR. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ significant difference with respect to controls; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ significant difference with respect to resilient mice. ###$p < 0.001$ significant difference with respect to day 5.

3.1.3. Susceptible mice showed altered levels of cytokine IL-6 and chemokine CX3CL1

The ANOVA for the striatal IL-6 levels showed an effect of the variable Stress [$F(2,37) = 9.957$; $p < 0.001$] (see Fig. 3a). Susceptible mice displayed higher IL-6 levels than the controls ($p < 0.001$), and resilient animals ($p < 0.01$). The ANOVA for IL-6 levels in PFC showed an effect of the variable Stress [$F(2,37) = 4.283$; $p < 0.021$] (see Fig. 3c). Susceptible mice displayed higher IL-6 levels than control animals ($p < 0.05$) without differences with resilient animals.

The ANOVA of striatal CX3CL1 levels revealed a significant effect of the variable Stress [$F(2,37) = 4.807$; $p < 0.014$] (see Fig. 3b). Striatal CX3CL1 levels were lower in susceptible animals in comparison with controls ($p < 0.01$). The ANOVA of CX3CL1 levels in PFC also revealed a significant effect of the variable Stress [$F(2,37) = 13.037$; $p < 0.007$] (see Fig. 3d). CX3CL1 levels in PFC were lower among all defeated animals (either resilient or susceptible) in comparison with controls ($p < 0.05$ for resilient and $p < 0.001$ for susceptible).

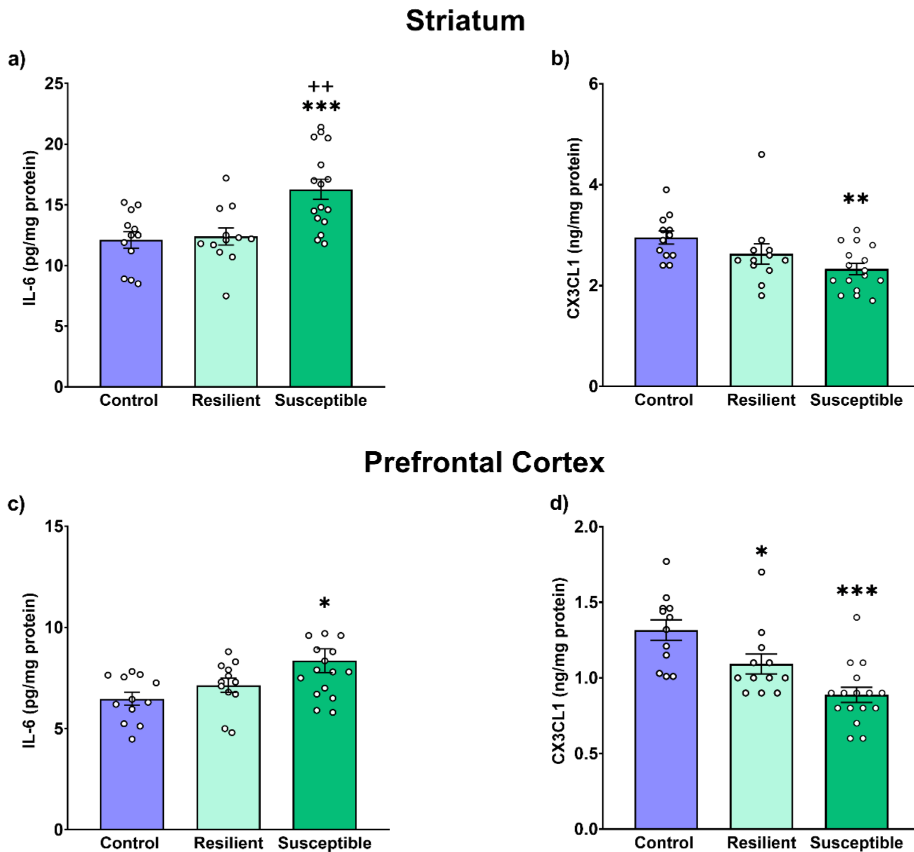

Fig. 3. Effect of repeated SD on IL-6 and CX3CL1 levels in the striatum and PFC. Bars represent mean pro-inflammatory cytokine IL-6 (in pg/mg) and chemokine CX3CL1 levels (in ng/mg) in the striatum (a and b) and PFC (c and d) and vertical lines ± SEM. Mice were divided into Control (n = 12); Resilient (n = 14) and Susceptible (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. *p < 0.05, **p < 0.01, ***p < 0.001 significant difference with respect to the control; ++ p < 0.01 significant difference with respect to resilient mice.

3.2. Environmental enrichment effects on resilience to SD

3.2.1. Environmental enrichment did not increase the percentage of resilient mice depending on the social withdrawal ratio

Following the SWR calculation criteria, the control group exposed to EE (n = 14) showed a mean SWR higher than 1.

In the defeated group of animals with EE (n = 31), 51.6% of mice showed a SWR under 1, which classifies them as susceptible mice (n = 16), and the remaining 48.4% of mice showed a SWR equal to or higher than 1, which classifies them as resilient mice (n = 15).

The comparison between the percentage of susceptible mice in the non-enriched experiment (53.3%) and the percentage of susceptible mice in the enriched experiment (51.6%) showed no statistical difference ($\chi2(1) = 0.018$; $p = 0.893$; Table 1).

Table 1. Housing condition and classification in the Social Interaction Test of defeated mice.

		Housing		Total
		Non-EE	**EE**	
Stress				
Susceptible mice	n	16	16	32
	%	53.3	51.6	52.5
Resilient mice	n	14	15	29
	%	46.7	48.4	47.5
Total	n	30	31	61
	%	100	100	100

3.2.2. Adolescent exposure to environmental enrichment reduces ethanol intake in susceptible animals

No differences were found between the animals during the training and substitution phases, showing that EE and SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Stress (Fig. 4a), with susceptible mice making more active responses than control animals ($p < 0.05$). During the FR3 schedule, the ANOVA revealed a significant effect of the variable Days [$F(4,168) = 4.215$; $p < 0.01$] (Fig. 4a). Mice performed less active responses on the 10th day compared to the 7th ($p < 0.01$), 8th ($p < 0.01$), and 9th ($p < 0.05$) days.

With respect to ethanol consumption, the ANOVA of the g/kg of ethanol intake during the FR1 and FR3 schedule of ethanol SA did not reveal any significant effect of the variable Days or Stress (Fig. 4b), meaning that defeated mice, either resilient or susceptible, did not consume more ethanol than non-stressed control animals.

During the PR, the ANOVA for the breaking point values of ethanol SA and for ethanol consumption did not reveal any significant effect of the variable Stress (Fig. 4c and d).

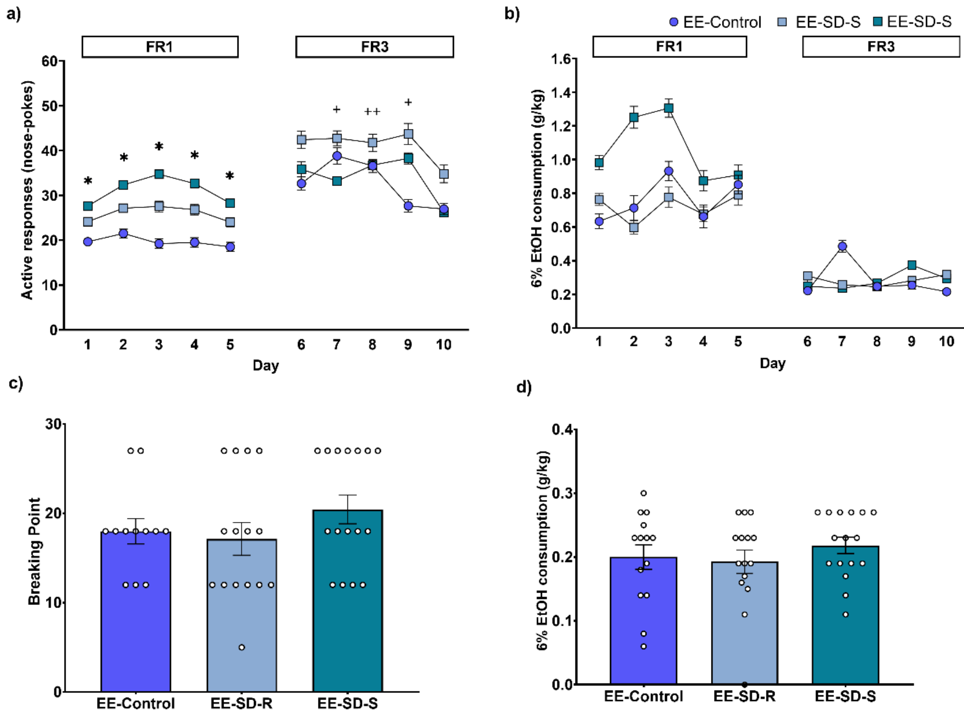

Fig. 4. Environmental enrichment reduces ethanol intake in susceptible animals. Mice were divided into EE-Control (n = 14); EE-SD-R (n = 15) and EE-SD-S (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% ethanol consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% ethanol consumption during PR. *$p < 0.05$, significant difference with respect to controls; + $p < 0.05$, ++ $p < 0.01$ significant difference with respect to the 10th day.

3.2.3. Environmental enrichment diminishes the neuroinflammatory response in susceptible mice.

The ANOVA of striatal IL-6 (Fig. 5a) and CX3CL1 (Fig. 5b) levels did not reveal any significant effect of the variable Stress.

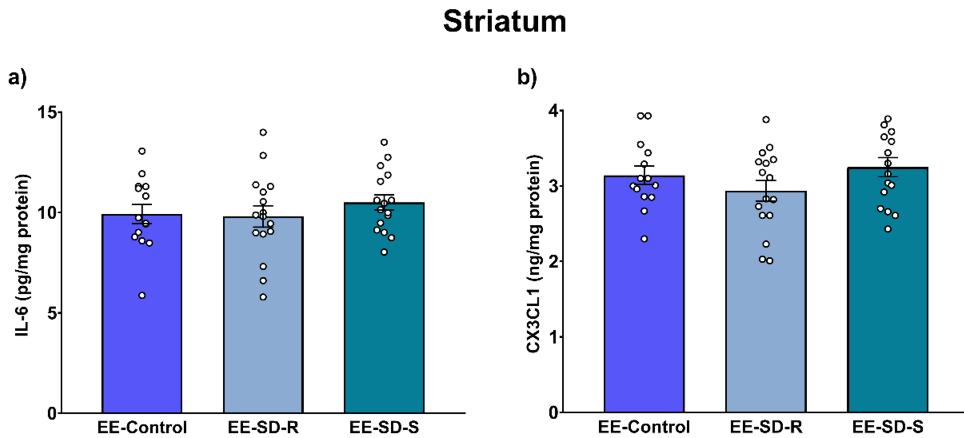

Fig. 5. Environmental enrichment reduces levels of the pro-inflammatory cytokine IL-6 and chemokine CX3CL1 in susceptible mice. Bars represent the mean of the striatal IL-6 (a) levels (in ng/kg) and CX3CL1 (b) levels (in pg/mg) and the vertical lines ± SEM. Mice were divided into EE-Control (n = 14); EE-SD-R (n = 15) and EE-SD-S (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR.

3.2.4. Environmental enrichment vs standard housing condition

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Housing [$F(1,78) = 4.451$; $p < 0.001$]. The post-hoc comparison showed that the enriched mice performed higher active responses than the non-enriched mice ($p < 0.05$). With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Housing [$F(1,75) = 6.050$; $p < 0.05$). The post-hoc comparison showed that the standard-housed mice consumed ethanol at higher rates than the enriched mice ($p < 0.05$).

During the FR3 schedule, the ANOVA revealed a significant effect of the interaction Days × Stress × Housing [$F(8,300) = 2.717$; $p < 0.01$) for the ethanol consumption. The standard housed group consumed significantly more ethanol than the control enriched group on day 6 ($p < 0.001$). The resilient standard-housed group consumed significantly more ethanol than the resilient enriched group on days 6, 7 (p's < 0.05),

8 ($p < 0.001$), 9 ($p < 0.01$) and 10 ($p < 0.001$). Moreover, the susceptible standard-housed group consumed significantly more ethanol than the susceptible enriched group on days 6, 7, 8, 9 and 10 (p's < 0.001).

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the interaction Stress × Housing [$F(2,79) = 3.052$; $p = 0.05$]. The post-hoc comparison showed that the breaking point values were higher in susceptible standard-housed mice with respect to the susceptible enriched animals ($p < 0.001$). The ANOVA for ethanol consumption during PR revealed a significant effect of the variable Housing [$F(1,81) = 65.766$; $p < 0.001$]. The post-hoc comparison showed that enriched mice consumed significantly less ethanol than standard-housed mice ($p < 0.001$).

The ANOVA for the striatal IL-6 levels showed an effect of the interaction Stress × Housing [$F(2,80) = 5.278$; $p < 0.01$]. The ANOVA revealed that the control standard-housed group displayed higher IL-6 levels than the control enriched group ($p < 0.05$). The susceptible standard-housed mice showed higher IL-6 levels than the susceptible enriched mice ($p < 0.001$). In the same line, the resilient standard-housed mice showed higher IL-6 levels than the resilient enriched mice ($p < 0.01$).

4. Discussion

It is well known that the behavioral and neurobiological effects of social stress are not equally manifested in all individuals. Most of the studies have focused on the particular response to depressive-like behaviors and neuroinflammation (Nasca et al., 2019; Pfau & Russo, 2015). However, few studies have evaluated the resilience/susceptibility response to drug abuse after social stress. We have recently reported that resilient mice to depressive-like behaviors also show a resilient response to the increased cocaine reward induced by SD, which is accompanied by a lower neuroinflammatory response (Ballestín et al., 2021). In the present study, we further confirm that mice presenting a phenotype resistant to depressive-like

behaviors are also unaffected by the increased ethanol intake induced by SD. Defeated resilient mice did not show any increase in ethanol intake, conversely to those susceptible, which also showed increased motivation for ethanol in the PR. These resilient mice developed minor neuroinflammatory responses with lower levels of IL-6 and higher levels of CX3CL1 in the PFC and the striatum than their susceptible counterparts. To further unravel the mechanisms of the resilient response, we evaluated the protective role of EE housing during adolescence before exposure to SD. Although EE during adolescence did not increase the percentage of resilient mice to depressive-like behaviors evaluated through the SWR, neither resilient nor susceptible mice increased their oral ethanol SA consumption. Moreover, none of the defeated mice exposed to EE developed any increase in neuroinflammatory markers. One limitation of the study is that the effects produced by SD in female rodents have not been evaluated in this work. Due to the sex differences observed in female mice with respect ethanol intake, it is necessary to perform suitable models of social stress for female rodents to address this issue in the future.

4.1. Resilience to the increase in ethanol intake induced by social defeat

The typical measure to classify animals as resilient or susceptible to SD effects is the SWR. Investigation of a social target is a natural behavior in healthy rodents; therefore, social avoidance is considered a depressive-like behavior. In the social interaction test, performed 24 h after the last SD, susceptible mice are stressed animals that display social avoidance.

Numerous studies have shown that SD induces changes in the reward system, affecting drug intake. With regard to ethanol, exposure to SD increases the conditioned rewarding effects of ethanol using the CPP paradigm (Macedo et al., 2018). Studies of voluntary ethanol consumption have observed increased and escalating consumption of ethanol, as well as an increased motivation to drink

alcohol, in defeated animals using the oral SA paradigm (Barchiesi et al., 2021; Montagud-Romero et al., 2021; Norman et al., 2015; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Using other paradigms such as the two-bottle choice, an increase in SD-induced escalation of alcohol intake has also been observed (Croft et al., 2005; Deal et al., 2018; Hwa et al., 2016; Newman et al., 2018a).

We have previously reported that animals classified as susceptible to SD-induced depressive-like behaviors were also susceptible to the increased rewarding effects of a subthreshold dose of cocaine three weeks after the last SD (Ballestín et al., 2021). In the present study, we confirmed that the resilient or susceptible phenotype to depressive-like behaviors induced by SD also correlates to the ethanol intake phenotype. In contrast to resilient mice, those classified as susceptible depending on the SWR test showed a higher increase in ethanol consumption and motivation to obtain a reward. There is only one other study evaluating this relation. Riga et al. (2020) evaluated the effects of a long-term SD-induced depressive phenotype, subsequently followed by a period of social isolation, on alcohol-seeking and drinking behaviors in male rats. This study presents significant differences with regards to ours, such as the use of two different social stressors, the SD and social isolation. The authors performed five SD exposures for five consecutive days and the intruder mice were immediately housed in isolation for the rest of the study. On the other hand, mice in our study were subjected to four intermittent sessions of SD and were socially housed throughout the study. It is known that the intensity, duration and number of exposures influence the intensity of subsequent behavioral symptoms and long-term effects on substance abuse (Shimamoto, 2018). Another important difference lies in the criterion to characterize the animals as resilient or susceptible to SD-induced depressive-like behaviors, which was based on social approach-avoidance and the object place recognition tests during the isolation period. Although these authors did not observe any increase in ethanol intake during the SA acquisition and FR1, susceptible rats exhibited a significant increase in

alcohol responsiveness during FR3 and a higher motivation to drink alcohol during the PR schedule compared to the control group. Susceptible rats also showed a higher number of extinction sessions and a higher relapse than non-stressed animals. Although no differences in ethanol consumption during FR1 were observed in the work of Riga et al. (2020), susceptible rats performed a higher number of active responses. One possible explanation for the lack of difference in ethanol intake could be the higher ethanol concentrations used (12%). We can hypothesize that SD may change the sensitivity to ethanol preference, with stressed animals being more sensitive to a low ethanol concentration, such as the 6% used in our study. In addition, social isolation is known to induce profound behavioral and neurobiological alterations (Mumtaz et al., 2018). Besides inducing anxiety and depressive-like behaviors in rodents (Amiri et al., 2015), several studies pointed that isolation induces an increase in ethanol consumption in mice and rats (Advani et al., 2007; Evans et al., 2020; Juárez & Vázquez-Cortés, 2003; Lopez et al., 2011; Sanna et al., 2011).

4.2. Susceptible mice showed increased levels of IL-6 and CX3CL1

Numerous studies have shown that ethanol activates the innate immune system by stimulating Toll-like receptor 4 (TLR4) signaling in glial cells, triggering the release of inflammatory mediators and causing neuroinflammation (Alfonso-Loeches et al., 2010; Ibáñez et al., 2019; Montesinos et al., 2016; Pascual et al., 2015). The induction of astrogliosis and microgliosis increases the release of cytokines (IL-1β, IL-6, IL-17, TNF-α) and the production of chemokines (MCP-1, MIP-1α, CX3CL1), causing brain damage in various brain structures such as the PFC, striatum, hippocampus and cerebellum (Alfonso-Loeches et al., 2010; Bachtell et al., 2015; Drew et al., 2015; Fernandez-Lizarbe et al., 2009; Guerri & Pascual, 2019; Pascual et al., 2011, 2018; Vetreno & Crews, 2015). In addition to the neuroinflammatory

response, alcohol exposure diminishes cell proliferation, migration, growth and differentiation, even causing cell death (Alfonso-Loeches & Guerri, 2011).

The brain areas analyzed in this study play a crucial role in the addictive cycle. On the one hand, the PFC controls subcortical regions to drive motivated behavior (Koya et al., 2009; West et al., 2014). The PFC consists of subregions that appear to mediate different aspects of the addiction cycle. For example, the prelimbic area (PrL) or dorsal area in rats projects preferentially to the Nucleus Accumbens (NAc) core, and the infralimbic (IL) or ventral area projects preferentially to the NAc shell (Heidbreder & Groenewegen, 2003; Ongür & Price, 2000). PrL appears to play a critical role in cue-elicited drug seeking (Lasseter et al., 2010), and IL appears to be primarily involved in inhibiting drug seeking (Peters et al., 2008). On the other hand, the striatum also consists of subnuclei involved in different stages of the addictive cycle. The ventral striatum (NAc) is associated with incentive salience pathways and salience attribution, i.e., it has been associated with the reinforcing actions of drugs of abuse (Koob, 2015; Koob & Volkow, 2010). While the dorsal striatum is related to habit formation (stimulus-response habit learning), and therefore, is key in the development of habitual compulsive drug use (Koob, 2015; Koob & Volkow, 2010).

Exposure to social stress promotes an increase in the neuroimmune response. Numerous preclinical studies show that SD is accompanied by the activation of neuroinflammatory events, including microglial activation and increased cytokine production (Calcia et al., 2016; Ferrer-Pérez et al., 2018; Finnell & Wood 2016; Montagud-Romero et al., 2021; Rodríguez-Arias et al., 2017, 2018; Wohleb et al., 2011, 2012, 2014). In addition, SD promotes the deterioration of the blood-brain barrier (BBB), and a decrease in the expression of the tight binding protein claudin-5, laminin and collagen-IV has been observed in the hippocampus and NAc (Menard et al., 2017; Rodríguez-Arias et al., 2017). Using the same procedure to induce SD as in the present study, we have observed that exposure to SD can induce a long-

lasting increase in the concentration of pro-inflammatory cytokines such as IL-6 in the PFC, striatum and hippocampus (Ballestín et al., 2021; Ferrer-Pérez et al., 2018) and a significant upregulation of the protein pro-inflammatory markers NFkBp-p65, IL-1β, IL-17 A and COX-2 in the striatum of male mice (Montagud-Romero et al., 2021). An increase of chemokines such as CX3CL1 and CXCL12 in the striatum and PFC of defeated mice has also been observed (Reguilón et al., 2020, 2021), although a decrease in CX3CL1 protein levels in the hippocampus and striatum (Ballestín et al., 2021; Montagud-Romero et al., 2020) has also been described using another strain of mice. Both pro-inflammatory and anti-inflammatory functions for CX3CL1of have been described (Mattison et al., 2013; Sheridan & Murphy, 2013; Zujovic et al., 2000), since the CX3CL1-CX3CR1 signaling has a neuroprotective function and maintains communication between neurons and microglia (Sheridan & Murphy, 2013). CX3CL1 seems to have anti-inflammatory effects mainly (Lyons et al., 2009; Zujovic et al., 2000), and an efficient CX3CL1 signaling between neurons and microglia appears to be critical for the protection of social stress-induced depressive-like behaviors. For example, CX3CR1 KO mice showed an exaggerated HPA axis response to social stress (Winkler et al., 2017).

Moreover, individual differences in the neuroinflammatory mechanisms observed after SD stress have been described. When characterized as susceptible to the depressive-like behaviors induced by SD, these animals showed increased levels of cytokines IL-6, MCP-1 or IL-1β (Hodes et al., 2014; Stewart et al., 2015; Wood et al., 2015), with an increase of anti-inflammatory cytokines IL-4 and IL-10 in resilient rodents (Hodes et al., 2014; Stewart et al., 2015). A recent study observed that exposure to chronic unpredictable mild stress triggered a significant increase in Nod-like receptor pyrin containing 3 (NLRP3) expression only in susceptible mice, but not in resilient mice. These changes were accompanied by altered levels of IL-1β expression (Yang et al., 2021). Increases in both the NLRP3 and IL-1β expressions are associated with the development of depressive-like behaviors (Felger & Lotrich,

2013; Raison & Miller, 2013). Moreover, chronic SD caused a significant decrease in cAMP levels in the NAc neurons of susceptible mice (Zhang et al., 2020), promoting BBB permeability. These results indicate that stress resilience may be associated with reduced pro-inflammatory signaling, and suggest that therapeutic treatment on these pathways could promote stress resilience (Yang et al., 2021). However, only a recent study from our laboratory evaluated if this neuroinflammatory response is also observed in mice susceptible to the increased cocaine reward induced by SD. We observed that these mice exhibited elevated neuroinflammatory levels of the pro-inflammatory cytokine IL-6 and a decrease in the chemokine CX3CL1 in the striatum and hippocampus after being exposed to SD (Ballestín et al., 2021). Moreover, striatal and hippocampal IL-6 levels continued to be elevated more than 5 weeks after the last SD in susceptible mice. In the present study, we have corroborated and extended these results. After oral ethanol SA, susceptible mice showed increased IL-6 levels in the striatum and PFC. In addition, a decreased CX3CL1 was equally observed in both structures after SA in susceptible mice, although resilient animals also showed a decreased CX3CL1 in the PFC. To our knowledge, this is the first study showing that animals susceptible to the increased rewarding effects of ethanol induced by SD showed a long-lasting increase in the neuroinflammatory response.

4.3. EE promotes resilience to the effects of SD on alcohol intake and the neuroinflammatory response

In the second study, mice were housed in an enriched environment during adolescence (PND21), but housed under standard housing conditions from the first SD (PND47) until the end of the experiment. In other words, our objective was to determine the existence of a protective effect of EE on depressive-like behavior and the long-term vulnerability to the rewarding effects of ethanol and the neuroimmune

response induced by SD. Our results confirmed the protective effect of EE in ethanol intake and in the neuroinflammatory response induced by SD.

EE has been typically associated with an improved well-being, increased cognitive function and a potentiation of stress resilience, and different models of EE have been used in order to reduce vulnerability to the detrimental effects of SD. However, the results observed in the literature are discrepant. In mice housed in EE and then subjected to 7 days of daily SD, an increase in aggressiveness and anxiety has been described, probably derived from a change in social stability (McQuaid et al., 2013a, 2013b). In these studies, EE not only did not decrease the neuroinflammatory response, but it even increased the corticotropin-releasing factor (CRF) levels in the PFC in both stressed and control mice.

Nevertheless, other studies found that EE is active in diminishing the neurobiological and behavioral effects induced by social stress, indicating that housing conditions may modulate the impact of external stressors. EE reduces acute and chronic stress-induced anxiety-like behaviors and cognitive impairments (Bahi, 2017; Cordner & Tamashiro, 2016; Dandi et al., 2018; Marianno et al., 2017). In addition, animals under EE housing show minor corticosterone increases and neuronal activation after a stressful experience (Branchi et al., 2013; Mesa-Gresa et al., 2016; Reichmann et al., 2013).

In contrast with the previously presented studies, we applied an EE prior to the exposure to SD to determine whether this housing condition during adolescence could potentiate the resilient response to depressive-like behavior and increase ethanol intake induced by this kind of stress. Our results showed that exposure to EE prior to SD does not influence SD-induced depressive-like behavior evaluated by SWR. In this way, we observed the same percentage of resilient and susceptible animals according to this score among those housed in EE when comparing with those from the first experiment housed under standard conditions. However, stressed

resilient and susceptible mice housed in EE during adolescence did not show any long-lasting increase in ethanol intake or motivation to get the drug after SD. A recent study of Seo et al. (2021) observed that early exposure to EE is capable of blocking depressive-like behaviors induced by chronic unpredictable stress when animals are housed under standard conditions. In addition, previous housing under EE prevented epigenetic changes induced by this stressor. Although, as in our study, mice were exposed to EE during adolescence, there are important methodological differences between both studies. In Seo's study, mice were housed in EE for a longer period, but more importantly, they used a different type of stressor named chronic unpredictable stress. Finally, we employed mice of the OF1 strain, which are particularly affected by SD due to their high territoriality. All these differences could be responsible for the discrepant results in EE in preventing depression-like behaviors. Despite the lack of a standardized EE model, we consider the model employed in this investigation to be promising. Exposure to EE during adolescence has favored and enhanced adaptive behaviors in the face of subsequent exposure to social stress.

The role of EE in reducing ethanol intake has been widely demonstrated. For example, Rodríguez-Ortega et al. (2018) proved that housing adult mice in EE reduces ethanol binge intake, and likewise, social and environmental enrichment reduced ethanol preference (Holgate et al., 2017). However, there are few studies evaluating the therapeutic potential of EE on the reinforcing and motivational effects of ethanol induced by SD. Bahi (2017) observed that the increased anxiety-like behavior, the increase in ethanol intake and the appearance of ethanol-induced CPP were buffered by exposure to EE conditions after the stress experience. In a more recent study, EE also proved to counteract social stress effects favoring the extinction of memories associated with ethanol and reducing reinstatement of drug seeking (Bahi & Dreyer, 2020).

EE also modulated the neuroinflammatory response induced by SD in the striatum. We did not observe any changes in IL-6 or CX3CL1 levels in the striatum in any of the groups evaluated. Differently with the first set of animals, susceptible mice housed in EE did not show any increase in IL-6 levels in the striatum, as observed in susceptible animals housed under standard housing conditions. Moreover, CX3CL1 levels also did not decrease in these mice, as it did in susceptible mice in the first study. As we did not observe any differences in the striatum of mice housed under EE conditions, the area most closely related to rewarding behavior, we did not analyze PFC. These results suggest that exposure to EE before SD reduces the impact of long-term social stress on the neuroinflammatory response, acting as a protective factor. There are no similar studies evaluating the neuroinflammatory response of social stress and ethanol consumption applying EE models, but our results are in line with studies evaluating the effect of EE on the neuroinflammatory response of social stress. Attenuations of the increase in IL-6 and IL-β1 in the prefrontal mRNA expression induced by moderate social stress have been observed in animals under EE conditions (McQuaid et al., 2018).

Among the beneficial effects of EE that could account for the protective effect on the increased ethanol intake and the neuroinflammatory response, we should highlight an increase in neurogenesis with an elevated expression of brain-derived neurotrophic factor (BDNF; Novkovic et al., 2015; Schloesser et al., 2010) and an enhanced synaptic and transcriptomic capacity (Hüttenrauch et al., 2016; Zhang et al., 2018). Exposure to EE during adolescence could also change the dynamics of social interaction, sensory processing and the mechanisms underlying baseline stress, with a decrease in CRHR1 genes and an increase in hippocampal CRHR2 observed in male rats housed in EE conditions (Kentner et al., 2018). Among other factors, facilitation in problem-solving ability and oxytocin immunoreactive responsiveness induced by EE in male rats must also be taken into consideration (Neal et al., 2018).

5. Conclusions

To sum up, our results corroborate that SD produces depressive-like behaviors, increased reinforcing and motivational effects of ethanol and induced greater neuroinflammatory response in susceptible mice, contrary to what occurs in resilient animals. The susceptible phenotype for depressive-like behaviors predicts the increased reinforcing and motivational effects of voluntary ethanol consumption and a larger neuroinflammatory response almost 2 months after the last SD exposure. In addition, we demonstrate that EE promotes the development of adaptive responses to social stress, indicating the importance of exposure to complex environments during adolescence.

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Study 4

Voluntary exercise pre-exposure during adolescence enhances resilience in rodents subjected to social defeat and ethanol self-administration in adulthood

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In preparation

Abstract

Stressful experiences induce depressive-like behaviors, reduce social interactions, and increase vulnerability to substance use disorders such as alcoholism. Using animal models, we have previously shown that exposure to social stress through social defeat produces a depressive phenotype, increased alcohol seeking and consumption, accompanied by activation of the neuroinflammatory response. However, not all animals subjected to social stress develop depressive-like symptoms or addictive behaviors. Therefore, the aim of this study was to enhance resilience in the most vulnerable animals through physical exercise. The effect of exposure to physical exercise during adolescence prior to exposure to social stress (4 sessions every 72 hours) during adulthood was evaluated. After 24 hours of the last defeat session, all mice performed the social interaction test (SIT) and were classified into resistant and susceptible to depressive-like behavior. Two weeks later, during adulthood, all mice were subjected to the drinking in the dark and oral ethanol self-administration paradigms. The animals were then sacrificed to assess BDNF levels in striatum and hippocampus. Mice classified as susceptible in the exercise condition showed resistance to increased ethanol intake in the oral self-administration protocol, showing similar consumption and motivation to the control and resilient groups. On the other hand, decreased BDNF levels were observed in susceptible mice in both experimental conditions compared to the control groups after ethanol SA. In conclusion, voluntary wheel running pre-exposure prevented increased consumption and motivation for ethanol induced by social defeat.

Keywords: Physical exercise; wheel running; social stress; resilience; ethanol; BDNF; self-administration

Abbreviations

BDNF: brain-derived neurotrophic factor; BP: breaking point; CPP: conditioned place preference; DA: dopamine; DID: drinking in the dark; ELISA: enzyme-linked immunosorbent assay; FR1: fixed ratio 1; HPA: hypothalamic-pituitary-adrenal; NAc: nucleus accumbens; PFC: prefrontal cortex; PR: progressive ratio; SA: self-administration; SD: social defeat; SIT: social interaction test; SWR: social withdrawal ratio; TBC: two-bottle choice; TH: tyrosine hydroxylase; VTA: ventral tegmental area; VWR: voluntary wheel running

1. Introduction

For decades, socially stressful experiences have been associated with the deterioration of mental health. A clear example is how social stress induces neuronal and behavioral alterations that increases vulnerability in the addictive process (Montagud-Romero et al., 2018; Newman et al., 2018; Nikulina et al., 2014; Vasconcelos et al., 2015). The scientific literature has related exposure to social stress with numerous modifications in the mesocorticolimbic system, being considered a risk factor in the process of transition from drug intake to addiction (Burke & Miczek, 2014, 2015; Nikulina et al., 2012; Wang et al., 2013). The resident-intruder paradigm, also known as social defeat, is a preclinical model validated for rodents and based on social hierarchy and dominance (Koolhaas et al., 2013; Miczek, 1979). Long-term defeat is associated with cognitive impairment, social deficits, anxiety, anhedonia, depressive-like behavior, and also increased vulnerability to drug use (Bath et al., 2017; Montagud-Romero et al., 2018; Stein et al., 2017). Exposure to social defeat (SD) in rodents causes a short and long increases and escalation of ethanol consumption (Norman et al., 2015; Reguilón et al., 2020, 2021a, 2021b; van Erp & Miczek, 2001). These changes in alcohol intake have been associated with social stress-induced neuroadaptations in hypothalamic, extrahypothalamic, and mesocorticolimbic circuits related to stress and reward (Holly et al., 2015; Hwa et al., 2016; Laine et al., 2017; Newman et al., 2018).

However, as in humans, not all rodents develop depressive-like behaviors or increased drug seeking and taking induced by SD. Resilience is the process and outcome of successfully adapting to difficult or challenging life experiences, especially through the flexibility of our body's neural circuitry and biological responses (for review, see Bath et al., 2017; Dantzer et al., 2018; Han & Nestler, 2017). In recent decades, several studies have focused on the neurobiological mechanisms involved in stress resilience. Resistant mice appear to have a different biological coping strategy than their more susceptible conspecifics. The

characterization of phenotypes resilient/susceptible to social stress allows training individuals to prevent the onset of problems such as anxiety and depression, and to reduce the risk of developing vulnerability to drug abuse. A resilient response has been associated with reduced reactivity of the HPA axis to chronic stress, with positive neuroadaptations in structures that form the limbic system, such as the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC), increased neurogenesis in the hippocampus, and a different immune response to susceptible animals (Ballestín et al., 2021; McEwen, 2016; Nasca et al., 2019; Reguilón et al., 2021b). In our laboratory, we have observed that mice resilient to SD-induced depressive-like behaviors showed a decrease in avoidance/flee and submissive/defensive behaviors compared to susceptible mice (Ballestín et al., 2021). In addition, resilient mice do not show an increase in conditioned and operant response to the rewarding effects of cocaine nor an increase in ethanol consumption induced by SD compared to susceptible mice (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). Also, we have observed that resilient mice showed lower increases in kynurenine concentration in the cerebellum, as well as, a reduced neuroinflammatory response, with decreases in IL-6 levels and increases in CX3CL1 levels in PFC, striatum and (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b).

The World Health Organization (WHO, 2020) report on physical activity shows that it reduces symptoms of depression and anxiety, and improves thinking, learning and judgment skills. In addition, the WHO emphasizes physical exercise at all stages of the life cycle, showing that in adolescence, physical activity improves cognitive outcomes (academic performance and executive function) and mental health (reduction of depressive symptoms). In preclinical studies, voluntary exercise in wheels has been shown to have a potentiating effect on learning and neurogenesis, resulting in increased neurotrophic factors and molecular signaling changes, as well as a reduction in depressive-like behaviors (Mul, 2018; Salam et al., 2009). Some

studies have shown that physical exercise regulates the hypothalamic-pituitary-adrenal (HPA) axis (Lynch et al., 2019). Prolonged exposure to physical exercise generated an adaptive response that decreased anxiogenic-type responses (Pietrelli et al., 2018). In addition, voluntary wheel running (VWR) had beneficial effects that prevented restraint stress-induced anxiety/depression behaviors and memory impairment (Lapmanee et al., 2017). Also, it has been observed that physical exercise during adolescence reduced serum corticosterone levels in the hippocampus induced by maternal separation in adult rats (Zolfaghari et al., 2021). Other studies focusing on the reward system have shown that physical exercise alters the transcription of genes in the mesolimbic reward pathway that induce changes in the rewarding properties of drugs of abuse and facilitate successful stress management (Greenwood et al., 2011). In relation to the rewarding effects of ethanol, physical exercise has been observed to significantly reduce ethanol consumption and preference in paradigms such as TBC and operant oral self-administration (SA; Darlington et al., 2014, 2016; Ehringer et al., 2009; Gallego et al., 2015; Reguilón et al., 2020).

Brain-derived neurotrophic factor (BDNF) is considered critical for neuronal and synaptic plasticity throughout the nervous system (Baj et al., 2012; Erickson et al., 2012; Liu & Nusslock, 2018; Vaynman et al., 2004). BDNF has been shown to be central in the development of addictive behavior and SD-induced depressive-like behaviors, as it is involved in reward circuitry, specifically in VTA dopaminergic neurons projecting into the NAc (for review, see Koo et al., 2019; Krishnan, 2014; Nikulina et al., 2014). Physical activity in different modalities (with or without load and over short and long distances) has great benefits on cognitive functions involving the hippocampus and BDNF signaling in the hippocampus (Lee et al., 2012). Physical exercise increases cortical, hippocampal and striatal BDNF expression in rodents subjected to physical and social stress, and at different periods of life,

resulting in increased neuroplasticity and prevention of neuronal death (Lee et al., 2012; Marais et al., 2009; Pietrelli et al., 2018; Sasse et al., 2013).

Previously, in our laboratory, we observed that controlled voluntary exercise during and after exposure to SD effectively reduced ethanol consumption and neuroinflammation associated with SD exposure in adult mice (Reguilón et al., 2020). Although there are studies that have evaluated the role of physical exercise and social stress, there are none that have studied the protective role of physical exercise during adolescence in enhancing resilience. We considered it necessary to explore the effects of physical exercise as a preventive method and as an enhancer of resilience development to the negative effects on ethanol consumption induced by SD. Potentially protective interventions during adolescence affect brain development and shape neural circuits that regulate later stress responses. Providing tools to counteract the adverse effects of SD is essential to reduce vulnerability to mental disorders such as depression or substance use disorder (El Rawas et al., 2020). Therefore, the aim of this study was to evaluate the role of physical exercise during adolescence as a preventive tool against the development of vulnerability to the rewarding and motivational effects of ethanol induced by social stress in stress-susceptible rodents. In addition, BDNF expression in hippocampus and striatum was evaluated, since physical exercise has been related to an increase in the expression of this neurotrophic factor that may influence these structures and modify the associative learning cues involved in the oral SA of ethanol and in the neuroplasticity associated with the learning of drug use (Baruch et al., 2004; Eisenstein & Holmes, 2007; Greenwood et al., 2011).

2. Methodology

2.1. Animals

A total number of 76 adult male C57BL/6 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. Experimental mice were

housed in groups of five in plastic cages (27×27×14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N=20) and were individually housed in plastic cages (21×32×20 cm) for at least one month prior to initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998a). All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (white light from 8:00 to 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (number 2017-VSC-PEA-00224, on December, 11th 2017).

2.2. Drugs

For the drinking in the dark and oral SA procedures, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a 20% (v/v) ethanol solution.

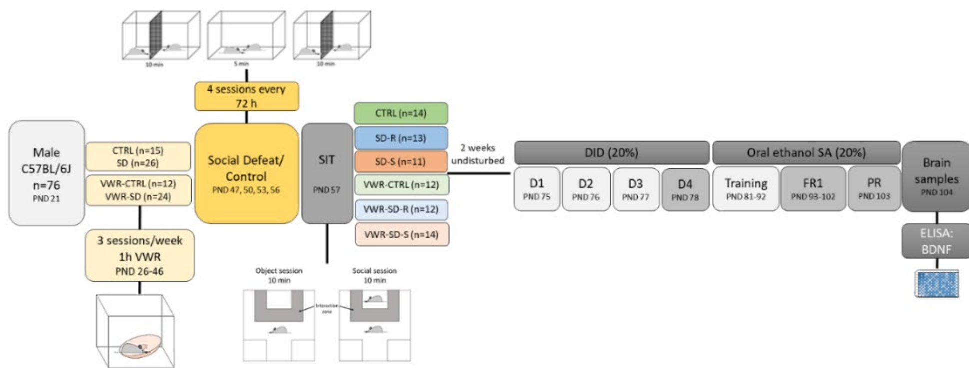
2.3. Experimental design

In the experimental design, all the animals were delivered to our laboratory PND 21 and were housed in regular condition throughout the study. Four experimental groups were randomly assigned, on the one hand, a control group and a defeated group (which remained undisturbed until the PND 47), and on the other hand, a control group and a defeated group which were exposed VWR 1h of exercise for 3 days a week from PND 26 to PND 46. Subsequently, all mice were exposed to the SD procedure or exploration from PND 47 to 56. 24 h after the last SD episode, animals performed the social interaction test (SIT) to evaluate depressive-like behaviors and were characterized as resilient or susceptible depending on their social withdrawal ratio (SWR). From this point on, the SD groups are divided into susceptible and resilient groups, forming 6 experimental groups: CTRL; SD-R; SD-

S; VWR-CTRL; VWR-SD-R; VWR-SD-S. Three weeks after the last defeat, the animals initiated the dinking in the dark (DID) test during four days and, in the following week, animals initiated the ethanol SA protocol for approximately 22 days. At the end of this test, all the animals were sacrificed to obtain the hippocampus and striatum for further analysis of the BDNF levels.

The experimental design is depicted in Figure 1.

Figure 1. Experimental design



2.4. Procedure and apparatus

2.4.1. Low-profile running wheel

The type of wheel used was the low-profile running wheel (Med Associates Inc.), which rotates on a central axis in a horizontal plane, allowing physical activity to be carried out through natural exercise as in spontaneous locomotion. These wheels have an ideal size (10.25x15.5x13.7) to be introduced into the home cages of rodents and are linked to a monitoring system (Hub) that runs on batteries and can register the activity through a set of programs (Wheel Manager Software).

2.4.2 Procedure of social defeat (SD)

Animals in the stress/defeated groups were exposed to 4 episodes of SD during adulthood, each lasting 25 min and consisting of three phases. The initial phase began by introducing the “intruder” (the experimental animal) into the home cage of the “resident” (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-minute period. Finally, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a “resident” mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al., 1998.

2.4.3. Social interaction test (SIT)

The SWR used was based on the social approach-avoidance test previously described by Berton et al., 2006. The test took place 24 h after the last SD during dark light cycle and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Animals were

allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10×6.5×35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Before each session, the arena was cleaned with 5 % alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Arena occupancy during object and social sessions were determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage. Corners were defined as two squares of similar areas on the opposite wall of the arena.

2.4.4. Drinking in the dark (DID)

Following the basic paradigm of Rhodes et al., 2005, the test consists of two phases. The first is habituation, where the animals are removed from their cages to be housed individually for one week to habituate them to the cages and the suction tubes containing a ball bearing at the end to prevent leakage, which will be used throughout the test. In the second phase of the protocol, the test begins 3 hours after lights out and the water bottles are replaced with 10 ml graduated cylinders containing a 20% (v/v) ethanol solution. These will remain in place for 2 hours. After this 2-hour period, the animals are returned to their grouped cages, with food and water bottles *ad libitum* again. This procedure is repeated on days 2 and 3, and on day 4, the procedure lasts for 4 h. In addition, immediately after each day, liquid consumption is recorded. Fresh ethanol solution is prepared each day. In our case, we will maintain the protocol for two consecutive weeks, one for habituation and one for testing.

2.4.5. Oral ethanol self-administration (SA)

This procedure is based on that employed by Navarrete et al., 2014. Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 20 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, fixed ratio 1 (FR1) and progressive ratio (PR) with a 20% ethanol concentration.

Training phase (12 days)

Mice were trained to respond to the active nose-poke to receive 20 µl of 20% (v/v) ethanol reinforcement. No food or water deprivation was performed in this protocol.

FR1 (10 days)

The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 20% ethanol (v/v) intake and the motivation to drink. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of effective responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 10 daily consecutive sessions.

PR (1 day)

A PR session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point (BP) value for this animal would be 40. All the sessions lasted one hour, except the PR session, which lasted two hours.

2.4.6. Immunoassay analysis (ELISA)

Samples from the hippocampus and striatum were obtained 24 hours after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and hippocampus and striatum dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Franklin & Paxinos, 2008; Heffner et al., 1980), which were then kept in dry ice until storage at -80ºC. Before BDNF determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al., 2010. Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of BDNF in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Biosensis, 211BEK-2211-1P). We determined BDNF concentration in the hippocampus and striatum. All reagents and standard dilutions were prepared following the manufacturer’s instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the final results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of picograms of BDNF. Data are expressed as pg/mg of protein for tissue samples.

2.5. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SWR. SWR is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups.

To analyze acquisition of ethanol SA, a three-way ANOVA was performed with two between-subjects variables –SD with three levels (CTRL, SD-S and SD-R); VWR with two levels (not exposed to VWR and exposed to VWR)– and a within-subjects variable –Days, with ten levels of FR1–. The effects of SD and VWR on breaking

point values and ethanol consumption during PR was analyzed by a one-way ANOVA, with two between-subjects variable –SD and VWR.

The data of the BDNF levels were analyzed using a one-way ANOVA with two between-subjects variables –SD, with three levels (Control, Resilient and Susceptible;) and VWR, with two levels (not exposed to VWR and exposed to VWR).

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics v.26. Data were expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Classification between susceptible and resilient mice according to their social withdrawal ratio

Following the SWR calculation criteria, the CTRL group (n=14) showed a mean SWR higher than 1. In the SD group of animals (n=26), 45.83% of the mice showed a SWR under 1, which classifies them as susceptible (SD-S) mice (n=11), and the remaining 54.17% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient (SD-R) mice (n=13).

Moreover, the VWR-CTRL group (n=12) showed a mean SWR higher than 1. In the VWR-SD group of animals (n=24), 53.85% of the mice showed a SWR under 1, which classifies them as susceptible (VWR-SD-S) mice (n=14), and the remaining 46.15% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient (VWR-SD-R) mice (n=12).

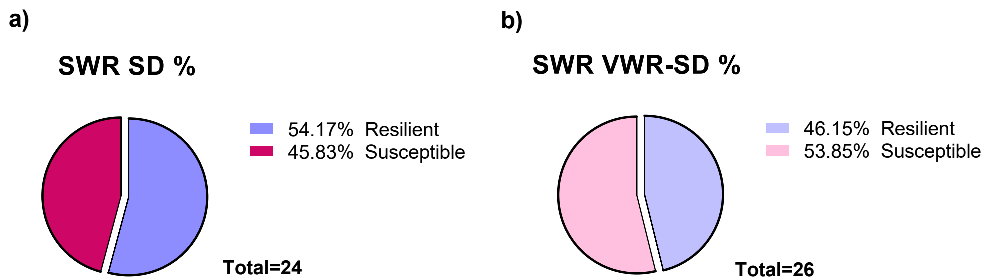


Figure 2. Percentages of resilient and susceptible animals among groups of animals defeated without running wheel access and with running wheel access during adolescence. The pie chart represents the percentage of resilient vs susceptible mice after SWR evaluation in the SIT.

3.2 Voluntary wheel running during adolescence decreased ethanol consumption in the DID paradigm

The ANOVA for ethanol consumption during DID paradigm revealed a significant effect of the variables Days [$F(3,180) = 41.249$; $p < 0.001$] and VWR [$F(1,60) = 50.448$; $p < 0.001$], and interaction Days × VWR [$F(3,180) = 11.482$; $p < 0.001$] (Fig.3). The post-hoc comparison showed that all animals consumed significantly more ethanol during day 4 compared to days 1, 2 and 3 (p's < 0.001), since on day 4th the test lasted 4h contrasting to 2 h during the first three days. Moreover, all animals trained in VWR consumed less ethanol during all DID procedure compared to untrained groups (p's < 0.001).

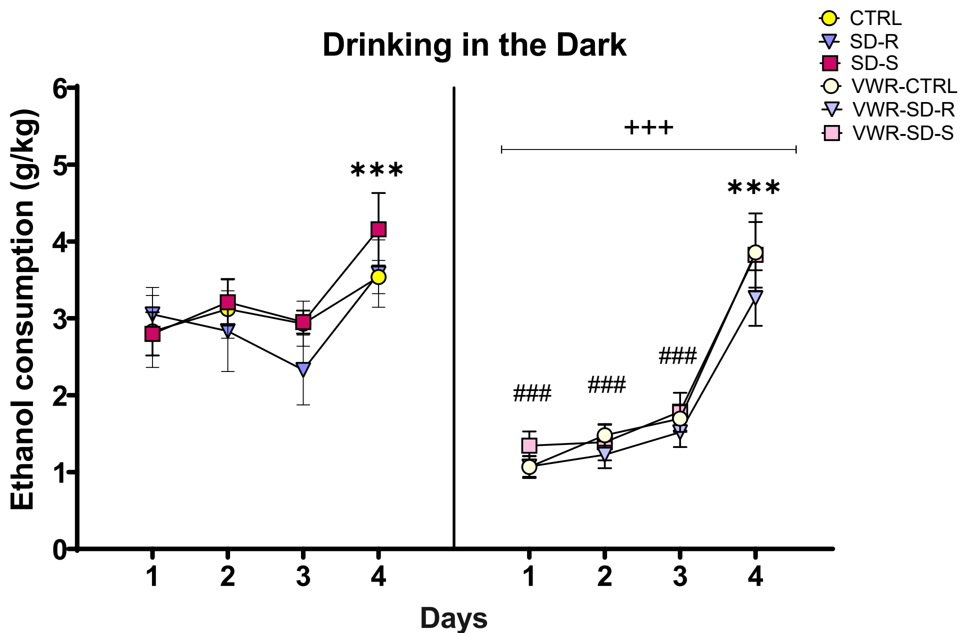


Figure 3. Effect of adolescent VWR on ethanol intake during DID. Animals were divided into the following six treatment groups: CTRL group allowed to explore a new cage and without access to a running wheel (CTRL, n = 14) or CTRL group allowed to explore a new cage and with access to a running wheel (CTRL-VWR, n = 12); Resilient SD group exposed to agonistic encounters and without access to a running wheel (SD-R, n=13) or Resilient SD group exposed to agonistic encounters and with access to a running wheel (SD-R-VWR, n=12); and Susceptible SD group exposed to agonistic encounters and without access to a running wheel (SD-S, n=11) or Susceptible SD group exposed to agonistic encounters and with access to a running wheel (SD-S-VWR, n=14). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of the g/kg of ethanol at 20% consumed. *** $p<0.001$ significant difference on day 4 compared to days 1, 2 and 3; ### $p<0.001$ significant difference between VWR groups vs. non-VWR groups on days 1, 2 and 3; +++ $p<0.001$ significant difference between VWR groups vs. non-VWR groups.

3.3 Susceptible animals that performed VWR show greater resistance to increased consumption and motivation for ethanol induced by social stress

No differences were found between the animals during training phase, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable SD [$F(2,70) = 4.140$; $p < 0.05$] and interactions Days × SD [$F(18,630) = 1.793$; $p < 0.05$]; Days × VWR [$F(18,630) = 2.158$; $p < 0.05$] (Fig. 4a). The post-hoc comparison showed that all animals classified as susceptible (SD-S and VWR-SD-S groups) performed more active responses compared to controls animals (CTRL and VWR-CTRL groups; $p < 0.05$). Specifically, these differences were observed on days 6 ($p < 0.05$), 7 ($p < 0.01$), 8 ($p < 0.01$), 9 ($p < 0.05$) and 10 ($p < 0.05$). It was also observed that the resilient animals (SD-R and VWR-SD-R groups) performed a greater number of effective responses than the control animals (CTRL and VWR-CTRL groups) on days 6 and 7 (p's < 0.05). Moreover, the post-hoc comparison showed the performance of a greater number of active responses in all groups of animals subjected to VWR compared to all groups not exposed to physical exercise on days 1, 4 and 5 (p's < 0.05).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the interactions Days × SD [$F(18,630) = 2.089$; $p < 0.01$) and SD × VWR [$F(2,70) = 6.142$; $p < 0.01$] (Fig. 4b). The post-hoc comparison showed that during day 1 resilient animals (SD-R and VWR-SD-R groups) consumed a significantly less amount of ethanol than during days 9 ($p < 0.05$) and 10 ($p < 0.01$). Also, all animals classified as susceptible (SD-S and VWR-SD-S groups) showed significantly less consumption during day 1 compared to days 6 ($p < 0.01$), 7 ($p < 0.05$), 8, 9 (p's < 0.001) and 10 ($p < 0.01$), during day 4 compared to days 6, 8 and 9 (p's < 0.01), and during day 5 compared day 8 ($p < 0.05$). Furthermore, the post-hoc comparison

showed that SD-S group consumed significantly more ethanol than the CTRL ($p < 0.001$), SD-R ($p < 0.01$) and VWR-SD-S ($p < 0.001$) groups.

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the interaction SD × VWR [$F(2,70) = 3.980$; $p < 0.05$] (Fig. 4c). The post-hoc comparisons showed that the SD-S group achieved a significantly higher BP than the CTRL ($p < 0.001$), SD-R ($p < 0.05$) and VWR-SD-S ($p < 0.05$) groups.

The ANOVA for ethanol consumption during PR revealed a significant effect of the interaction SD × VWR [$F(2,70) = 3.105$; $p < 0.05$] (Fig.4d). The post-hoc comparisons showed that SD-S group consumed significantly higher amounts of ethanol compared to CTRL ($p < 0.01$), SD-R ($p < 0.05$) and VWR-SD-S groups ($p < 0.01$).

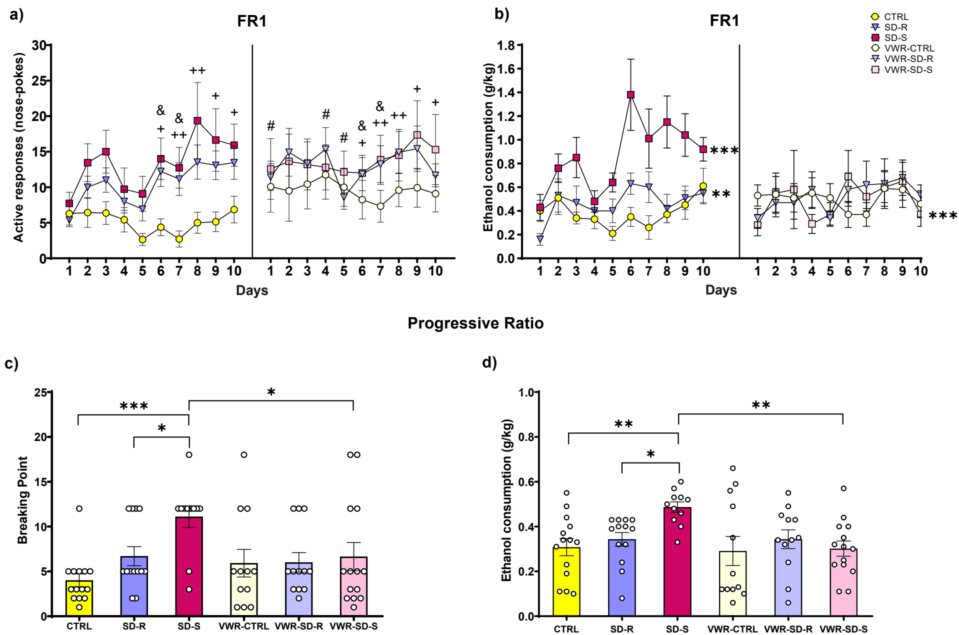


Figure 4. Effects of running wheel on the increase in oral ethanol self-administration induced by social stress in C57BL/6J mice. Animals were divided into the following six treatment groups: CTRL group allowed to explore a new cage and without access to a running wheel (CTRL, n = 14) or CTRL group allowed to explore a new cage and with access to a running wheel (CTRL-VWR, n = 12); Resilient SD group exposed to agonistic encounters and without access to a running wheel (SD-R, n=13) or Resilient SD group exposed to agonistic encounters and with access to a running wheel (SD-R-VWR, n=12); and Susceptible SD group exposed to agonistic encounters and without access to a running wheel (SD-S, n=11) or Susceptible SD group exposed to agonistic encounters and with access to a running wheel (SD-S-VWR, n=14). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the gr/kg of ethanol at 20% consumed during FR1 schedule. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, and (d) the gr/kg of ethanol at 20% consumed during PR. ***$p < 0.001$, ** $p<0.01$, * $p<0.05$ significant difference with CTRL or SD-S groups; ++ $p< 0.01$, + $p<0.05$ significant difference between susceptible mice vs. controls; & $p<0.05$ significant

difference between resilient mice vs. controls; # $p<0.05$ significant difference between groups with running wheel access vs. groups without access.

3.4 Social defeat and ethanol exposure induce decreased BDNF levels in all susceptible mice

The ANOVA revealed a significant effect of the variable SD [$F_{(2,68)}=4.125$; $p < 0.05$] on BDNF protein levels after oral SA of ethanol in striatum (Fig. 5a). Post-hoc comparison revealed that a significant decrease in BDNF protein levels was observed in all susceptible mice according with their SIT (SD-S and VWR-SD-S groups) compared to controls (CTRL and VWR-CTRL groups) in striatum ($p < 0.05$).

Moreover, the ANOVA revealed a significant effect of the variable SD [$F_{(2,67)}=3.653$; $p < 0.05$] on BDNF protein levels after oral SA of ethanol in hippocampus (Fig. 5a). Post-hoc comparison revealed that a significant decrease in BDNF protein levels was observed in all susceptible mice according with their SIT (SD-S and VWR-SD-S groups) compared to controls (CTRL and VWR-CTRL groups) in hippocampus ($p < 0.05$).

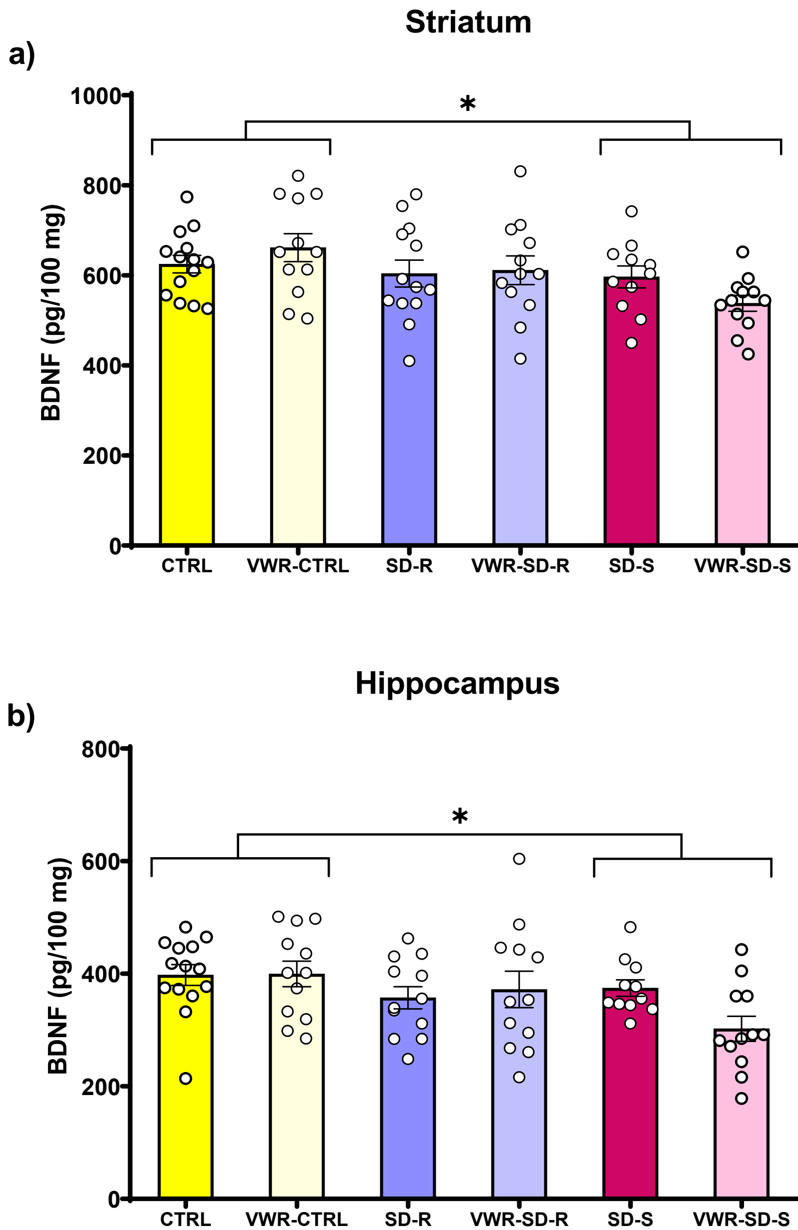


Figure 5. Effects of long-term social defeat on BDNF protein levels in C57BL/6J mice.
Animals were divided into the following six treatment groups: CTRL group allowed to

explore a new cage and without access to a running wheel (CTRL, n = 14) or CTRL group allowed to explore a new cage and with access to a running wheel (VWR-CTRL, n = 12); Resilient SD group exposed to agonistic encounters and without access to a running wheel (SD-R, n=12-13) or Resilient SD group exposed to agonistic encounters and with access to a running wheel (VWR-SD-R, n=12); and Susceptible SD group exposed to agonistic encounters and without access to a running wheel (SD-S, n=11) or Susceptible SD group exposed to agonistic encounters and with access to a running wheel (VWR-SD-S, n=12). Defeated mice were characterized as resilient or susceptible depending on their SIT. The columns represent the mean and the vertical lines ± SEM of the BDNF protein levels (pg/100 mg of protein) in (a) striatum and (b) hippocampus. * $p<0.05$ significant difference between susceptible mice (SD-S and VWR-SD-S groups) vs. controls mice (CTRL and VWR-CTRL groups).

4. Discussion

Resilience is a complex phenomenon with an innate character that can be developed and enhanced with various strategies such as the promotion of physical activity (Belcher et al., 2021; McEwen, 2006; Southwick et al., 2005). In the present study we have corroborated that there are two populations of defeated mice. Mice with a susceptible phenotype, which developed social avoidance in the SIT and shoed increased ethanol intake, and resilient mice that did not alter their social contacts nor the ethanol consumption. The most important result of the present study is to prove the preventive effect of voluntary and controlled physical exercise during adolescence prior to the exposure of SD. Although VWR during adolescence did not increase the percentage of resilient mice to depressive-like behavior, those susceptible did not show the increase in alcohol consumption and preference in adulthood. Therefore, physical exercise during adolescence was able to reverse the long-term behavioral consequences on ethanol induced by social stress. However, WVR during adolescence did not counteract the decrease in striatal and hippocampal levels of BDNF in susceptible mice.

Voluntary wheel running did not influence the development of the resilient phenotype

The SIT is an etiologically validated and widely used model that allows classification of the resilient phenotype or susceptibility to SD-induced depressive-like behaviors as a function of rodent social avoidance behavior (Berton et al., 2006; Cathomas et al., 2019; Golden et al., 2011; Krishnan et al., 2007). In this study, exposure to physical activity during adolescence and prior to experience SD did not affect the percentage of resilient animals according to SIT. We reported 46% of resilient mice after exposure to the running wheel versus 54% of resilient mice that did not have access to the running wheel. In a previous study in our laboratory, we also did not observe that environmental enrichment prior to social stress increased the percentage of resilient mice, although it was an effective intervention to avoid the increased ethanol consumption and neuroinflammatory response associated with the long-term negative consequences induced by SD (Reguilón et al., 2021b). Access to the running wheel prior to a forced swim stress, reduced anxious behaviors but did not affect the endocrine response, with similar increased in corticosterone levels after stress (Lynch et al., 2019). Recently, Zhang et al. (2021) suggested that the dopaminergic system might be involved in the development of susceptible and resistant phenotypes to SD. They performed a classification of mice according to distance traveled, mice with low baseline physical exercise showed susceptibility to SD and mice with high baseline physical exercise showed resistance to the consequences of SD in the SIT. Mice with low baseline physical exercise showed lower expression of tyrosine hydroxylase (TH) protein and TH-positive neurons than mice with high baseline physical exercise. Activation of TH neurons in the VTA using the designer receptor exclusively activated by designer drugs (DREADDs) method increased physical activity time and resilience to SD (Zhang et al., 2021).

Despite the lack of consensus, multiple studies claim that physical exercise is beneficial on mental disorders related to social stress. In various models of SD, physical exercise has been observed to produce resistance to the development of social avoidance behaviors, anhedonia (Mul, 2018; Pagliusi et al., 2020; Zhang et al., 2019), hyperalgesia (Pagliusi et al., 2020), increased neuroinflammatory response (Reguilón et al., 2020) and in the decrease of TH levels in VTA and D2 receptor in the NAc shell (Zhang et al., 2019). We should specify that exposure to VWR has followed a different pattern in each of these studies. On the one hand, physical exercise has been used as a preventive strategy, exposing rodents before and/or during SD (Mul, 2018; Pagliusi et al., 2020; Reguilón et al., 2020), and, on the other hand, as a strategy to reverse the effects of SD, exposing rodents to the wheel after SD (Pagliusi et al., 2020; Zhang et al., 2019).

Physical activity reduces alcohol consumption

It is well established that SD produces long-term changes on reward pathways, increasing ethanol preference and consumption (Miczek et al., 2015; Pautassi et al., 2010; Reguilón et al., 2020, 2021a, 2021b; Rodriguez-Arias et al., 2016). In the present study we have corroborated this SD effect on ethanol consumption. Susceptible mice to depressive-like behaviors showed significantly higher consumption than resilient and control mice during the FR1 schedule of oral ethanol SA. Furthermore, during the PR, susceptible mice to depressive-like behaviors consumed more ethanol and obtained a higher BP than control and resilient mice to depressive-like behaviors, indicating a higher motivation to obtain the substance. In contrast, susceptible mice to depressive-like behaviors that were exposed to VWR prior to SD showed significantly lower alcohol consumption than the susceptible group not exposed to physical exercise during the DID and FR1 schedule of the oral ethanol SA. In addition, differences in BP and consumption during the PR were observed as susceptible mice with VWR showed lower motivation and alcohol

consumption during the PR compared to the susceptible group that did not perform physical exercise.

Although access to VWR during adolescence prior to SD did not affect the resilient/susceptible phenotype in SIT, it did have a protective effect on the long-term increased ethanol consumption in susceptible mice. Previously in our laboratory, we have obtained similar results with adult male mice exposed to physical exercise after SD (Reguilón et al., 2020). Besides to decreased alcohol consumption, WVR decreased neuroinflammatory response when compared to defeated mice without access to physical exercise.

There are several studies that confirmed the protective role of physical exercise on ethanol intake. Using the TBC paradigm, several studies have observed lower consumption in rodents of both sexes with access to physical exercise compared to sedentary rodents (Booher et al., 2019; Darlington et al., 2014, 2016; Ehringer et al., 2009; Gallego et al., 2015; Hammer et al., 2010; Piza-Palma et al., 2014). In another study of similar characteristics, adolescent mice were subjected to controlled physical exercise (5.5 h per day) for 14 days and immediately after this period were exposed to an alcohol binge model, a reduction in behavioral sensitivity to alcohol intoxication and neuroprotection against alcohol-induced cell death in granule cells in the dentate gyrus of the hippocampus were observed (Leasure & Nixon, 2010). However, in our study we have apply the exercise during adolescence, weeks before than the ethanol intake will take place.

Many studies suggest that exercise with running wheels have a reinforcing effect and lead to neuroplastic changes in the reward pathway. It appears that acute physical exercise influences the activity of monoamines such as serotonin, dopamine and norepinephrine (Gomez-Merino et al., 2001; Lin & Kuo, 2013). Long-term exercise induces adaptations in serotonergic 1A receptors in the raphe nuclei and dopamine receptor D2 in the striatum (Bauer et al., 2020; Clark et al., 2015; Greenwood, 2019).

Adaptations in these monoamines induced by physical exercise may alter the response of these monoamines during ethanol intake and lead to decreased vulnerability to develop problematic alcohol consumption (Buhr et al., 2021). Similar to drugs of abuse, VWR has been shown to induce increased levels of ΔFosB in the NAc (Greenwood et al., 2011; Herrera et al., 2016; Werme et al., 2002) and dorsal striatum (Herrera et al., 2016), increased activity of DA neurons in the lateral VTA (Herrera et al., 2016), increased levels of TH mRNA in the VTA, delta opioid receptor in the NAc shell, and decreased levels of DA receptor D2 mRNA in the NAc core (Greenwood et al., 2011) and Drd1a mRNA expression in striatum (Darlington et al., 2014). Access to wheels for at least 6 weeks was also shown to be rewarding and induce conditioning place preference (CPP; Greenwood et al., 2011; Herrera et al., 2016). These results indicate that VWR has rewarding properties, altering gene transcription in the mesolimbic reward pathway and may modify neurotransmitter responses to substance abuse and social stress exposure. Additionally, and based on the study by (Lespine & Tirelli, 2018), where 3 weeks of wheel running exercise during adolescence attenuated the onset and expression of cocaine sensitization in adult mice, we hypothesize that adolescence is a particularly sensitive period that may promote long-term resistance to the development of vulnerability to substance abuse use through voluntary physical activity.

Social defeat and ethanol consumption induced a decrease in BDNF levels in susceptible mice

The neurotrophin BDNF is widely distributed in the central and peripheral nervous system. It is of great importance for brain development and increased brain plasticity throughout life. The control of survival, growth and differentiation of specific neuronal populations are among the major functions of BDNF (Barde et al., 1987; Park & Poo, 2013; Zagrebelsky & Korte, 2014). BDNF has been widely implicated in the development of mood and addictive disorders (Bandelow et al., 2017;

Feltenstein & See, 2013; Lüscher & Malenka, 2011; Nestler, 2013). SD in rodents results in increased BDNF expression in several brain areas such as the PFC (Ferrer-Pérez et al., 2019), amygdala, bed nucleus of the stria terminalis (Vasconcelos et al., 2021) NAc and VTA (Berton et al., 2006; Fanous et al., 2010; Koo et al., 2019; Krishnan et al., 2007; Miczek et al., 2011). Increased BDNF expression in the mesolimbic pathway is widely associated with depressive-like behaviors and social avoidance (Krishnan, 2014; Nikulina et al., 2014). In contrast, SD-induced decreases in BDNF expression have been observed in the hippocampus (Guan et al., 2021; Martin et al., 2017; Yao et al., 2021). Intermittent ethanol exposure also produces increases in BDNF expression in mPFC (Montesinos et al., 2016; Somkuwar et al., 2016; Yang et al., 2017). However, when alcohol exposure is chronic the opposite effect has been observed, with a decrease in BDNF expression in dorsal striatum, mPFC and hippocampus (Logrip et al., 2015; Silva-Peña et al., 2019; Yang et al., 2017).

The performance of physical exercise on VWR produces transcriptional and post-transcriptional regulation of BDNF in different brain areas (Venezia et al., 2016; Cotman & Engesser-Cesar, 2002). In the hippocampus, increases in BDNF expression have been observed in adult and adolescent male and female rodents from 6 days to 28 weeks of training (Gallego et al., 2015; Griesbach et al., 2004; Lee & Soya, 2017; Seifert et al., 2010; Venezia et al., 2016). Increases in frontal cortex have also been observed after 3 weeks of physical exercise in adult male rats (Graban et al., 2017).

Among the benefits of VWR, the most studied is the effect it has on anxious- and depressive-like behaviors in rodents (for a review, see Mul, 2018). There are no studies evaluating BDNF in rodents subjected to SD pre-exposed to running wheels. Some studies in physical stress (restraint) demonstrate that 3 to 6 weeks of voluntary physical activity prior to stress counteracts decreases in BDNF expression in the

hippocampus (Adlard & Cotman, 2004; Greenwood et al., 2007; Lapmanee et al., 2017). In relation to alcohol, discrepancies have been observed. On the one hand, VWR for 21 days accompanied by exposure to alcohol by TBC, produced an increase of BDNF expression in the hippocampus (Gallego et al., 2015). While in another study after 16 days of physical exercise and alcohol exposure, a decrease in BDNF expression in the hippocampus was observed compared to mice in physical exercise condition without alcohol exposure (Darlington et al., 2014).

In the present study we did not observed any effect of WVR on BDNF level. A decrease in BDNF protein levels in both striatum and hippocampus was observed in susceptible mice with and without access to running wheels compared to the control groups (CTRL and CTRL-VWR). This lack of effect could be justify considering the experimental procedure. There was a period of 9 weeks from the last exercise with VWR to the analysis of BDNF levels. If exercise caused an increase in BDNF in these structures, after 9 weeks it would probably have returned to normal levels, since BDNF levels induced by physical activity usually decrease after 14 days of wheel removal (Berchtold et al., 2010). On the other hand, alcohol exposure induces BNDF increases in hippocampus after acute alcohol exposure but can produce decreases after chronic exposure (Logrip et al., 2015; Silva-Peña et al., 2019; Yang et al., 2017). Therefore, we can hypothesize that alcohol exposure after SD induced changes in BDNF signaling with decreased levels in the striatum and hippocampus only in susceptible mice. BDNF signaling could be considered as a mediator of stress, as increases in BDNF have been observed only in VTA in SD-susceptible mice (Krishnan et al., 2007), suppression of this neurotrophin in VTA promoted resilience to social stress (Berton et al., 2006) and increases in BDNF levels in the hippocampus have been observed in rats resilient to the chronic mild stress paradigm (Bergström et al., 2008). Considering these data, we must recognize a limitation of the present study since we did not analyze BDNF levels at different time points of

the experimental procedure, although a great number of animals have had been employed.

5. Conclusions

Our results suggest that voluntary, controlled physical exercise during adolescence is a beneficial environmental intervention with long-term power to promote resilience to future ethanol exposure. Encouraging healthy physical activity habits seems to be beneficial for the correct regulation of the mesolimbic system, both in relation to an adaptive response to social stress and to protection against the development of problematic alcohol consumption.

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Study 5

Stress inoculation in adolescence: attenuation of the rewarding and motivational effects of social stress-induced ethanol in male mice

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In preparation

Abstract

Exposure to social stress is associated with an increased risk of developing a substance abuse disorder. Stressful experiences during adolescence can affect brain development, thus exerting a profound and lasting influence on mental development and psychological health. In this research we set out to study a hypothesis related to early-life stress exposure that assumes that individuals who have experienced moderate intensity stress may acquire some resilience to deeper stress exposure in adulthood. We evaluated the effect of a single exposure to social defeat (SD) during adolescence on the increased voluntary ethanol consumption induced by exposure to repeated and intermittent social stress during adulthood. During adolescence, half of the animals were subjected to a single SD exposure (postnatal day 28). Three weeks later, (postnatal day 49) defeated groups were subjected to 4 confrontation sessions with an aggressive resident at 72h intervals, while the control groups were exposed to non-resident exploration sessions. 24h after the last SD, the defeated mice were classified in resilient or susceptible depending on their response to a social interaction test (SIT, depressive-like behavior model). To assess voluntary consumption and motivation for ethanol, two weeks after the 4 SD experience during young adulthood, drinking in the dark and oral ethanol self-administration paradigms (20%) were used. The results showed that stress inoculation (SI) slightly increased the percentage of resilient animals in the SIT. As previously observed, susceptible defeated mice showed an increase consumption and motivation for ethanol with respect the control and resilient mice. Stress inoculation (SI) during adolescence efficiently blocked this effect as no increase in ethanol intake in SI-SD groups was observed irrelevant of being classified as resilient or susceptible. These groups also showed similar motivation than controls to obtain ethanol measured by the PR. Interestingly, the increased in IL-6 levels observed in SD-S mice was absent in those exposed to SI. Moreover, those groups exposed to SI presented lower PFC levels of

IL-6 and CX3CL1. Therefore, our data support the hypothesis that SI, through exposure to low-intensity stress during adolescence, may enhance resilience to other higher intensity stressors in adulthood.

Keywords: stress inoculation; social defeat; resilience; susceptibility; ethanol; IL-6; CX3CL1

Abbreviations:

BP: breaking point; CRF: cortocitropin-releasing factor; CTRL: control; DID: drinking in the dark; ELISA: enzyme-linked immunosorbent assay; FR1: fixed ratio 1; HPA: hypothalamic- pituitary- adrenal; PFC: prefrontal cortex; PND: postnatal day; PR: progressive ratio; SA: self-administration; SD: social defeat; SI: stress inoculation; SIT: social interaction test; SUD: substance used disorders; SWR: social withdrawal ratio

1. Introduction

It is widely documented that exposure to intense stressors in adult life is associated with the development of addictive behaviors, such as alcoholism. Circumstances that can be aggravated if unfavorable situations have been suffered during early stages of life (Loman & Gunnar, 2010; Lucassen et al., 2013). Adolescence is a critical period of biological, behavioral and social changes. These changes together with family and social experiences can be a risk factor for adverse behaviors and situations in adulthood (Crews et al., 2007; Forbes & Dahl, 2010). Adverse situations during adolescence have been strongly associated with the onset of mental illness in adulthood, such as the development of anxiety and depression, as well as being related to substance used disorders (SUD) and an increase in violent behaviors (Ho & King, 2021; Lee et al., 2012).

In preclinical studies, the social defeat (SD) model, described by Miczek (1979), represents an etiologically validated model for the study of the consequences of social stress. This model induces long-term physiological and behavioral changes consistent with the characteristic symptoms of anxiety and depression (Iñiguez et al., 2014; Macedo et al., 2018), and produces modifications in important neurotransmitter systems (Burke & Miczek, 2015), vulnerability to drug addiction (Burke & Miczek, 2015; Macedo et al., 2018; Montagud-Romero et al., 2018) and leads to an increase in the immune response (Ballestín et al., 2021; Reguilón et al., 2021a, 2021b).

Despite the adverse effects that social stress can have on individuals, not everyone develops these negative effects. In both humans and animal models, the phenomenon of resilience has been observed in which complex neurobiological mechanisms have been involved are very complex (Cathomas et al., 2019; Feder et al., 2019; Murrough & Russo, 2019; Russo et al., 2012; Wood & Bhatnagar, 2015). Rodents resistant to the adverse effects of SD during encounters showed proactive behaviors during SD,

resist to show defeat/submission behaviors and develop adaptive changes in corticotropin-releasing factor (CRF) signaling, such as habituation of the hypothalamic-pituitary-adrenal (HPA) axis with decreased CRF efficacy and optimal activity of the dopaminergic system (Ballestín et al., 2021; Friedman et al., 2014; Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Ródenas-González et al., 2020; Wood et al., 2010). The innate immune system has also been associated with the resilient response to social stress. Subjects resistant to the effects of SD show a normalized immune response compared to susceptible subjects, who conversely increase inflammatory cells and pro-inflammatory mediators (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Hodes et al., 2014; Pfau et al., 2019; Reguilón et al., 2021a, 2022; Ródenas-González et al., 2020).

Resilience to social stress is a dynamic response, as we have recently showed in adolescent mice exposed to SD (Reguilón et al., 2022). Differently from adults, that exhibited a consistent resilient phenotype to depressive- and increased drug effects after SD, adolescent defeated mice showed a partial resilient phenotype with approximately 20% showing full resilient phenotype. These results support the idea that resilience is a dynamic response que evolve through the life. In this line, we have efficiently potentiated resilience by housing adolescent mice in an environmental enrichment prior exposure to a SD protocol in adulthood (Reguilón et al., 2021a). A more challenging approach points to mild-to-moderate stressors during early life periods as a tool to strength resilient response. If a stressful experience exceeded the own capabilities, it tends to be considered as a threat, out of control and negatively affects emotion and behavior. However, if the individual can deal with the adverse situation, it tends to be considered as a challenge (Southwick & Charney, 2012). In recent decades, several models and strategies have been developed to promote resilience in animal models. Brief maternal separation, limitation of nesting and bedding material during early rodent life stages, exposure to predator odor, or interaction with an aggressive male during adulthood (for a review, see Ashokan et

al., 2016). Positive results have been obtained after mild exposure to predator odors during adulthood (Wang et al., 2020); sensory contact during a social agonistic encounter during adulthood (Ayash et al., 2020; Brockhurst et al., 2015); limited nesting and bedding material paradigm (Hsiao et al., 2016). Finally, one of the most widely used paradigms for inducing early stress experiences is the maternal separation (for a review, see Alves et al., 2020). Moderate maternal separation prevented the increase in anxious behaviors and decreased neural excitability in basolateral amygdala in mice subjected to chronic SD during adulthood (Qin et al., 2019). In the same way, brief maternal separation has been reported to increase resilience to cocaine-conditioned reward effects induced by repeated SD in late adolescence (Calpe-López et al., 2022). The above-mentioned studies have been mostly performed in adult animals or during pre-weaning period, but there are no studies evaluating the long-term effects of SI during adolescence.

Protective interventions during adolescence affect brain development and shape neural circuits regulating future responses to stress. Providing tools to counteract the adverse effects of social stress is essential to reduce vulnerability to mental disorders such as depression or SUD (EL Rawas et al., 2020). In this study we aimed to enhance resilience to the negative effects of adult exposure to SD by a moderate-intensity exposure to social stress during adolescence. We hypothesized that this intervention will induce a protective effect against exposure to more profound stressful situations during adulthood. To this end, we performed SI by exposing mice to a single SD during adolescence. Subsequently, the animals were subjected to 4 SD sessions during young adulthood, and 24h later, all animals will be classified according to their depressive-like behavior using the social interaction test (SIT). Three weeks later, ethanol sensitivity was assessed using the DID and oral SA paradigms. Finally, the neuroinflammatory response was determined by analyzing striatal and cortical levels of the pro inflammatory cytokine IL-6 and the chemokine CX3CL1.

2. Methodology

2.1. Animals

A total number of 77 adult male C57BL/6 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. Experimental mice were housed in groups of five in plastic cages (27×27×14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N=20) and were individually housed in plastic cages (21×32×20 cm) for at least one month prior to initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998b). All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (red light from 8:00 to 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (number 2017-VSC-PEA-00224, on December, 11th 2017).

2.2. Drugs

For the drinking in the dark (DID) and oral SA procedures, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a 20% (v/v) ethanol solution.

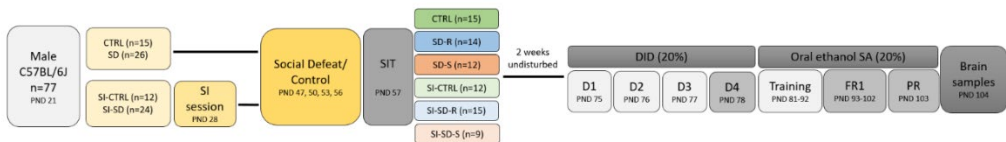
2.3. Experimental design

In the experimental design, all the animals were delivered to our laboratory PND 21 and were housed in regular condition throughout the study. Four experimental groups were randomly assigned, on the one hand, a control group and a defeated group (which remained undisturbed until the PND 47), and on the other hand, a control group and a defeated group which were exposed to a single SD encounter in PND 28. Subsequently, all mice were exposed to the SD procedure or exploration

from PND 47 to 56. 24 h after the last SD episode, animals performed the social interaction test (SIT) to evaluate depressive-like behaviors and were characterized as resilient or susceptible depending on their social withdrawal ratio (SWR). From this point on, the SD groups are divided into susceptible and resilient groups, forming 6 experimental groups: CTRL; SD-R; SD-S; SI-CTRL; SI-SD-R; SI-SD-S. Three weeks after the last defeat, the animals initiated the DID test during four days and, in the following week, animals initiated the ethanol SA protocol for approximately 22 days. At the end of this test, all the animals were sacrificed to obtain the PFC and striatum for further analysis of the cytokine and chemokine levels.

The experimental design is depicted in Figure 1.

Figure 1. Experimental design



2.4. Procedure and apparatus

2.4.1. Brief stress inoculation (SI)

Animals randomly distributed to the SI-CTRL and SI-SD groups were subjected to exposure to a single SD at PND 28. This agonistic encounter was conducted following the same protocol as the SD detailed below in section 2.4.2. The only difference is that only one encounter was conducted during adolescence stage.

2.4.2 Procedure of social defeat (SD)

Animals in the stress/defeated groups were exposed to 4 episodes of SD during adulthood, each lasting 25 min and consisting of three phases. The initial phase

began by introducing the “intruder” (the experimental animal) into the home cage of the “resident” (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-minute period. Finally, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a “resident” mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al., 1998.

2.4.3. Social interaction test (SIT)

The SWR used was based on the social approach-avoidance test previously described by Berton et al., 2006. The test took place 24 h after the last SD during the dark cycle and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Animals were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10×6.5×35 cm) was placed in the middle of one wall of the arena. In the second

session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Before each session, the arena was cleaned with 5 % alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Arena occupancy during object and social sessions was determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage. Corners were defined as two squares of similar areas on the opposite wall of the arena.

2.4.4. Drinking in the dark (DID)

Following the basic paradigm of Rhodes et al. (2005), the test consists of two phases. The first is habituation, where the animals are removed from their cages to be housed individually for 2 h per day for one week to habituate them to the cages and the suction tubes containing a ball bearing at the end to prevent leakage, which will be used throughout the test. In the second phase of the protocol, the test begins 3h after lights out and the water bottles are replaced with 10 ml graduated cylinders containing a 20% (v/v) ethanol solution. These will remain in place for 2 h. After this 2 h period, the animals are returned to their grouped cages, with food and water bottles *ad libitum* again. This procedure is repeated on days 2 and 3, and on day 4, the procedure lasts for 4 h. In addition, immediately after each day, liquid consumption is recorded. Fresh ethanol solution is prepared each day. In our case, we will maintain the protocol for two consecutive weeks, one for habituation and one for testing.

2.4.5. Oral ethanol self-administration (SA)

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 20 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, fixed ratio 1 (FR1) and progressive ratio (PR) with a 20% ethanol concentration.

Training phase (12 days)

Mice were trained to respond to the active nose-poke to receive 20 µl of 20% (v/v) ethanol reinforcement. No food or water deprivation was performed in this protocol.

FR1 (10 days)

The aim of this phase was to evaluate the number of responses on the active nose-poke, the 20% ethanol (v/v) intake and the motivation to drink. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during this phase, the number of effective responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 10 daily consecutive sessions.

PR (1 day)

A PR session was completed to establish the breaking point (BP) for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the BP was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the BP value for this animal would be 40. All the sessions lasted one hour, except the PR session, which lasted two hours.

2.5. Immunoassay analysis (ELISA)

Samples from the striatum and the PFC were obtained 24 hours after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Franklin & Paxinos, 2008; Heffner et al., 1980), which were then kept in dry ice until storage at -80ºC. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). We determined CX3CL1 and IL-6 concentration in the striatum and PFC. All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the final results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

2.6. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SWR. SWR is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups.

To analyze acquisition of ethanol SA, a three-way ANOVA was performed with two between-subjects variables –SD with three levels (CTRL, SD-S and SD-R); SI with two levels (not exposed to SI and exposed to SI)– and a within-subjects variable – Days, with ten levels of FR1–. The effects of SD and SI on BP values and ethanol

consumption during PR was analyzed by a one-way ANOVA, with two between-subjects variable –SD and SI.

The data of the CX3CL1 and IL-6 levels were analyzed using a one-way ANOVA with two between-subjects variable –SD, with three levels (Control, Resilient and Susceptible) and SI, with two levels (not exposed to SI and exposed to SI).

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics (v.26; IBM, NY, USA) for behavioral data and GraphPad Prism (v8; GraphPad Software Inc., CA, USA) for graph design. Data were expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Classification between susceptible and resilient mice according to their social withdrawal ratio

Following the SWR calculation criteria, the CTRL group (n=15) showed a mean SWR higher than 1. In the SD group of animals (n=26), 46.15% of the mice showed a SWR under 1, which classifies them as susceptible (SD-S) mice (n=12), and the remaining 53.85% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient (SD-R) mice (n=14).

Equally, the SI-CTRL group (n=12) showed a mean SWR higher than 1. In the SI-SD group of animals (n=24), 37.5% of the mice showed a SWR under 1, which classifies them as susceptible (SI-SD-S) mice (n=9), and the remaining 62.5% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient (SI-SD-R) mice (n=15).

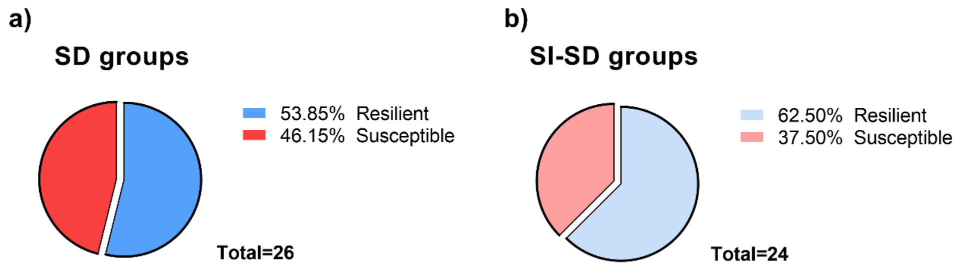


Figure 2. Percentages of resilient and susceptible animals among groups of animals defeated without stress inoculation and those subjected to stress inoculation during adolescence. The pie chart represents the percentage of resilient vs susceptible mice after SWR evaluation in the SIT.

3.2 Stress inoculation during adolescence leads to decreased ethanol consumption in the DID procedure

The ANOVA for ethanol consumption revealed a significant effect of the variable Days [$F(3,204) = 48.232$; $p < 0.001$], and the interaction SD × SI [$F(2,68) = 3.681$; $p < 0.05$] (Fig.3). The post-hoc comparison showed that all animals consumed a higher ethanol on day 4 compared to days 1, 2 and 3 (p's < 0.001 in all cases), since on day 4th the test lasted 4h contrasting to 2 h during the first three days. In addition, the SD-S group consumed higher amounts of ethanol than the SD-R ($p < 0.01$) and SI-SD-S ($p < 0.001$) groups during the DID test.

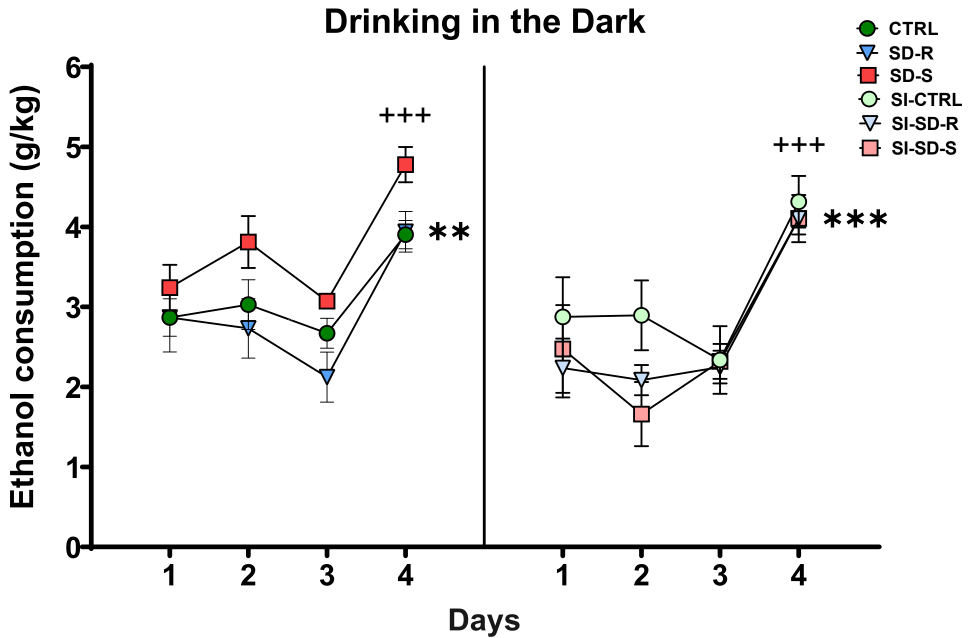


Figure 3. Effect of adolescent stress inoculation on ethanol intake during DID. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 15) or CTRL group exposed to SI in adolescence, but not exposed to SD in adulthood (SI-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n=14) or Resilient SD exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-R, n=15); and Susceptible SD group exposed to SD in adulthood (SD-S, n=12) or Susceptible SD group exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-S, n=9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of the g/kg of ethanol at 20% consumed. ***p 0.001, ** p<0. significant differences between the SD-S group and the SD-R and SI-SD-S groups.; +++ p<0.001 significant difference on day 4 compared to days 1, 2 and 3.

3.3 Stress inoculation during adolescence leads to decreased ethanol consumption in susceptible mice

No differences were found between the animals during training phase, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Days [$F(9,639) = 3.694$; $p < 0.001$], SD [$F(2,71) = 4.333$; $p < 0.05$], and the interaction Days × SD × SI [$F(18,639) = 2.317$; $p < 0.01$] (Fig. 4a). The post-hoc comparison revealed that mice performed fewer active responses on day 1 compared to days 2 ($p < 0.001$),3 ($p < 0.01$),4 ($p< 0.05$), 8, and 10 ($p < 0.01$ in all cases). Moreover, SD-S group performed more active responses than the CTRL group on days 3, 6 (p's < 0.05), 7, 8, 9 and 10 ($p < 0.01$). SD-S group also performed more active responses than SD-R group on day 7 ($p < 0.05$). Equally, SI-SD-S group performed more active responses on day 4 compared to the SI-CTRL ($p < 0.05$) and SI-SD-R ($p < 0.05$) groups. Finally, the post-hoc comparison showed a significant decrease in active responses in the SI-SD-R group on day 8 compared to the SD-R group ($p < 0.05$).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Days [$F(9,630) = 2.976$; $p < 0.01$) and a significant effect of the interaction SD × SI [$F(2,70) = 3.558$; $p < 0.05$] (Fig. 4b). The post-hoc comparison showed that during day 10 all animals consumed a significantly higher amount of ethanol than during days 1 ($p < 0.01$) and 4 ($p < 0.05$). Also, the SD-S group showed significantly higher consumption than the CTRL ($p < 0.001$), SD-R ($p < 0.01$) and SI-SD-S ($p < 0.01$) groups.

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the interaction SD × SI [$F(2,71) = 4.710$; $p < 0.05$] (Fig. 4c). The post-hoc comparisons showed that the SD-S group achieved a significantly higher BP than the CTRL ($p < 0.001$) and SD-R ($p < 0.05$) groups, while no significant

differences were observed between the groups exposed to a brief social defeat. Moreover, a higher BP was observed in the SI-CTRL group compared to the CTRL ($p < 0.05$) group, and a higher BP was observed in the SD-S group compared to the SI-SD-S ($p < 0.05$) group.

The ANOVA for ethanol consumption during PR revealed a significant effect of the interaction SD × SI [$F(2,71) = 4.298$; $p < 0.05$] (Fig.4d). The post-hoc comparisons showed that the SD-S group had a higher ethanol consumption than the CTRL ($p < 0.05$) and SI-SD-S ($p < 0.05$) groups, although SI-CTRL group consumed significantly more ethanol than the CTRL group ($p < 0.05$).

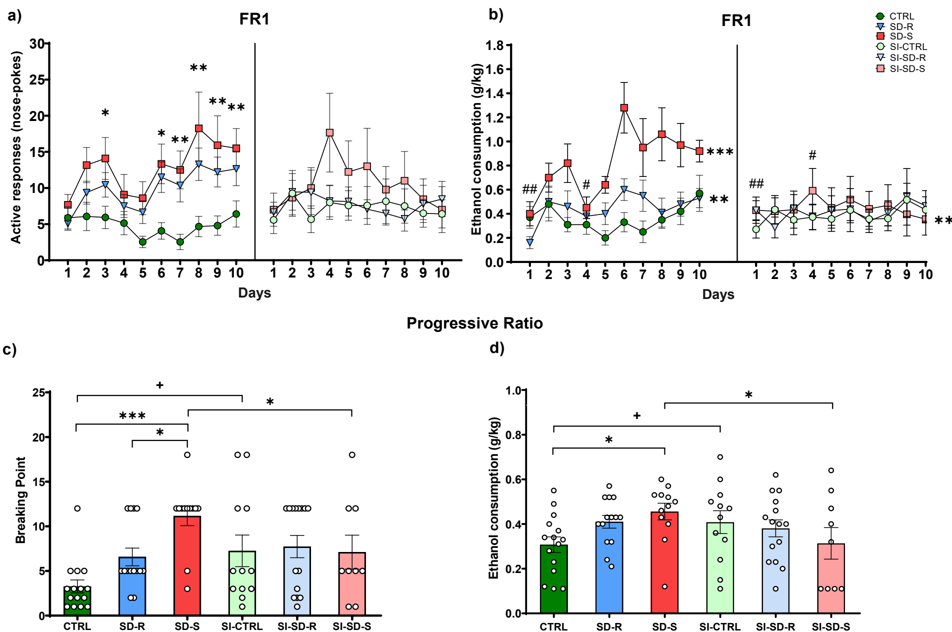


Figure 4. Effects of stress inoculation during adolescence on the increase in oral ethanol self-administration induced by social stress in C57BL/6J mice. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 15) or CTRL group exposed to SI in adolescence, but not exposed to SD in adulthood (SI-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n=14) or Resilient

SD exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-R, n=15); and Susceptible SD group exposed to SD in adulthood (SD-S, n=12) or Susceptible SD group exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-S, n=9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the g/kg of ethanol at 20% consumed during FR1 schedule. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, and (d) the g/kg of ethanol at 20% consumed during PR. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ significant difference with respect CTRL or SD-R; + $p<0.05$ significant difference between CTRL vs. SI-CTRL; ## $p<0.01$, # $p<0.05$ significant differences with day 10.

3.4 Stress inoculation during adolescence leads to decreased neuroinflammatory response in susceptible mice

The ANOVA for IL-6 levels in PFC showed an effect of the variable SI [$F(1,68) = 15.821$; $p < 0.001$] (see Fig. 5a). Post-hoc comparisons showed lower IL-6 levels in the SI-exposed mice (SI-CTRL, SI-SD-R and SI-SD-S groups) compared to non-SI-exposed mice (CTRL, SD-R and SD-S groups; $p < 0.001$). Moreover, the ANOVA for the striatal IL-6 levels showed an effect of the interaction SD × SI [$F(2,70) = 3.173$; $p < 0.05$] (see Fig. 5c). A significant increase in IL-6 levels was observed in the SD-S group compared to the CTRL ($p < 0.01$) and SD-R groups ($p < 0.05$). Additionally, both SD-S and SD-R groups showed higher IL-6 striatal levels than the corresponding groups exposed to SI ($p < 0.001$ for SI-SD-S, and $p < 0.01$ for SI-SD-R).

The ANOVA of CX3CL1 levels in PFC also revealed a significant effect of the interaction SD × SI [$F(2,70) = 5.760$; $p < 0.01$] (see Fig. 5b). The post-hoc comparisons revealed a significant increase in CX3CL1 levels in SD-S group compared to CTRL and SI-SD-S groups ($p < 0.01$ in all cases). Moreover, the results showed an increase in CX3CL1 levels in the SI-CTRL group compared to the CTRL

group (p < 0.05). The ANOVA of striatal CX3CL1 levels revealed no significant differences.

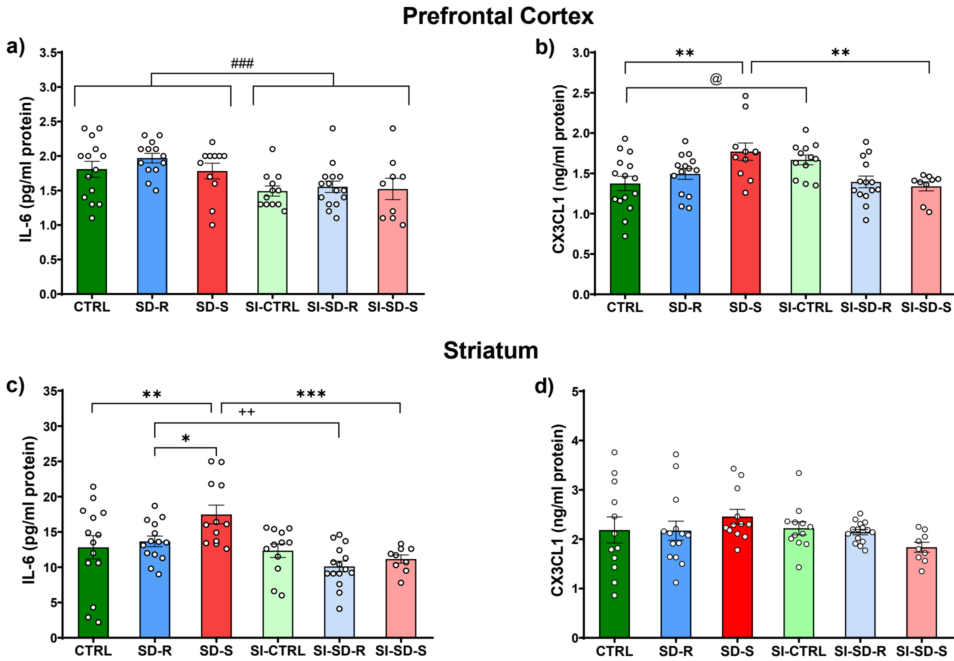


Figure 5. Effects of stress inoculation during adolescence in the neuroinflammatory response induced by social stress in C57BL/6J mice. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 12-15) or CTRL group exposed to SI in adolescence, but not exposed to SD in adulthood (SI-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n=13-14) or Resilient SD exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-R, n=15); and Susceptible SD group exposed to SD in adulthood (SD-S, n=11-12) or Susceptible SD group exposed to SI in adolescence and exposed to SD in adulthood (SI-SD-S, n=9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The columns represent the mean and the vertical lines ± SEM of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6, and of the striatal (b) and cortical (d) levels (in ng/mg) of the chemokine CX3CL1. ***p < 0.001, **p < 0.01, *p < 0.05 significant difference with

SD-S group; $++p < 0.01$ significant difference with SD-R group; @ $p < 0.05$ significant difference between CTRL vs. SI-CTRL; ### $p < 0.001$ significant difference between SI-exposed mice and non-SI-exposed mice.

4. Discussion

Resilience is a complex phenomenon that highlights individual differences. Behind this adaptive behavior there are many neurobiological mechanisms that are expressed in more effective coping strategies (Seery et al., 2010). These mechanisms are still being studied and involve changes in hormones, neurotransmitter systems, the immune response, and epigenetic changes, existing evidence of a genetic predisposition to this resilient phenotype. Few studies have evaluated the resilience/susceptibility response to drug abuse after social stress and how to enhance resilience to this stress effect. We already know that mice resilient to depressive-like behaviors also show a resilient response to SD-induced cocaine and ethanol reward enhancement, which is accompanied by a lower neuroinflammatory response (Ballestín et al., 2021; Reguilón et al., 2021a). In addition, we report the protective and resilience-enhancing value of environmentally enriched housing during adolescence prior to exposure to SD (Reguilón et al., 2021a). Experience of a mild controlled stress previous to a more intense stressful experience, or SI have been proved to also potentiate resilience. However, these studies have been performed in pre-weaning or adult ages, no studies made to date during adolescence.

In the present study, we corroborated that SD induced an increase in ethanol consumption alone in mice susceptible to depressive-like behaviors and an increase in protein levels of IL-6 in the striatum and CX3CL1 in the PFC. Further, our results showed that exposure to a unique episode of SD during adolescence buffers the negative effects of intermittent SD experience during adulthood. We observed that SI during adolescence resulted in a 9% increase in the resilient phenotype to depressive-like behaviors evaluated with the SIT. In relation to ethanol consumption during DID, SI blocked the increased consumption observed in mice susceptible to

depressive-like behaviors. The same result was observed during the FR1 schedule of oral ethanol SA and the PR schedule. We also observed a decreased neuroinflammatory response in all animals exposed to SI with reduced IL-6 levels in PFC and decreased IL-6 in the striatum of susceptible mice subjected to SI, as well as, decreased levels of CX3CL1 in PFC. In contrast, we observed increased consumption and motivation of control mice exposed to SI during the SA PR schedule compared to the non-inoculated control group, as well as increased neuroinflammatory response with increased levels of IL-6 in the PFC.

Defeated mice exposed to stress inoculation during adolescence showed increased social interaction in adulthood.

Using the SIT, a percentage of resilient mice around 50% among defeated mice is usually observed in different studies, despite the methodological differences in the social defeat procedure (Alves-dos-Santos et al., 2020; Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Ródenas-González et al., 2020). We have also observed similar percentage in mice under environmental enrichment conditions during adolescence (Reguilón et al., 2021a). Nevertheless, in the case of animals exposed to SI during adolescence we have observed that this percentage was increased. As shown in Figure 2, the percentage of animals resilient to the effects of SD after SI (62.5%) is higher than the resilient defeated animals without any intervention (53.8%).

There are few studies evaluating the impact of SI on social interaction in adulthood. In the line of our results, Ayash et al. (2020) observed that a significant increase in social interaction in SIT in SI-exposed mice compared to controls. Furthermore, they performed the SIT again 24h after acute SD and observed that this trend was maintained. These authors observed that SI-exposed mice spent significantly more time interacting with the social object than the control group. Although in the study by Hsiao et al. (2016), SI employed were different as the authors used the limited

nesting and bedding material paradigm, a significant increase in the proportion of mice resilient to chronic SD-induced depressive-like behaviors in adolescents was also observed. In the group of mice exposed to SI, the percentage of resilient mice after SIT was 60% versus 33% of resilient mice in the control group. In contrast, using the brief maternal separation model (6h during PND 9), no increase in social interaction was observed after 24h of repeated SD in late adolescence (Calpe-López et al., 2022). The authors hypothesize that perhaps the brief maternal separation time used is not sufficient to obtain the protective effect of SI during this early stage of life.

In the present study, the increase in resilient subpopulation in SI-exposed mice was only a 9% increase compared with resilient mice not exposed to SI. Although there is no consensus in the scientific literature, we hypothesize that SI might increase adequate adaptation to social stress in terms of the development of depressive-like behaviors in adulthood.

Stress inoculation enhances resilience to the increased ethanol consumption induced by social defeat

The literature has depicted multiple changes in the reward system induced by social stress that affect the reinforcing and motivational effects of ethanol. There is consensus that SD induced increased in voluntary ethanol intake and escalation in consumption, as well as an increase in motivation to obtain the substance (Barchiesi et al., 2021; Croft et al., 2005; Deal et al., 2018; Hwa et al., 2016; Montagud-Romero et al., 2021; Newman et al., 2018; Norman et al., 2015; Reguilón et al., 2020, 2021b; Rodriguez-Arias et al., 2016). In previous studies we observed that the phenotype resilient to SD-induced depressive-like behaviors also showed resilience to the increased drug effects (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Ródenas-González et al., 2020). Therefore, the results obtained in this study corroborate that mice with a phenotype susceptible to depressive-like

behaviors also increased consumption of and motivation for ethanol and that conversely, mice resilient to depressive-like behaviors did not show this increase in ethanol intake (Reguilón et al., 2021a). Relative to SI-exposed mice, we firstly observed that the percentage of the animals resilient to SD-induced depressive-like behavior was higher (9% increased) than the percentage of resilient animals not exposed to SI. More importantly, defeated susceptible mice exposed to SI (37,5%) did not show an increase in ethanol consumption. Therefore, in addition to an increase in resilience to depressive-like behaviors, SI blocked SD-induced increase in ethanol intake irrelevant of the response observed in social behaviors. Despite that the DID paradigm was used as a pre-exposure to ethanol, the analyses of the dinking behavior of the mice during the procedure indicated that SD-S group consumed more ethanol than the other groups, e.g. more than SD-R and SI-SD-S groups. Following this line, results obtained during FR1 schedule of oral ethanol SA, susceptible mice previously inoculated with stress (SI-SD-S) consumed similar amounts as resilient mice (SD-R and SI-SD-R) and the CTRL group, and substantially lower amounts than susceptible mice not exposed to SI (SD-S). Equally, higher motivation to obtain the drug, with higher breakpoint and ethanol consumption during the progressive SA schedule was only observed in the SD-S groups. Interestingly, it was also observed that the control group exposed to SI (SI-CTRL) performed higher ethanol consumption and showed a higher breakpoint compared to the control group without SI exposure (CTRL) during the PR schedule. This suggests that exposure to a single SD during adolescence may create certain vulnerability to ethanol consumption during adulthood (for review, see Camarini et al., 2018).

Although, there are no similar studies to compare our results, based on the evidence that SI promotes resilience to future stressful experiences, we hypothesize that SI could be a helpful intervention in order to reduce the increased in substance use related to stressful experiences. In a recent study, a preventive effect of brief maternal separation on the long-term effects of subsequent SD on vulnerability to

cocaine reward has been observed in mice (Calpe-López et al., 2022). Similarly, Ordoñes Sanchez et al. (2021), used the limited bedding and nesting model of adversity to assess SI on phenotypes related to opiate addiction in adulthood. Male rats subjected to SI showed reduced impulsive choice, and self-administered less morphine with reduced motivation to obtain the substance. In the nucleus accumbens, SI dampened glutamate transmission and blocked morphine-induced plasticity. In summary, the few studies performed to date indicated that SI during early life can elicit a resilient effect against addiction-related phenotypes. An important flaw of this conclusion is that is based only in studies performed in male animals, no studies of the of SI been performed in females. In conclusion, more research is needed to study the role of SI on the effects of subsequent stressful experiences and on addictive behavior in adulthood in both sexes.

Stress inoculation reduces neuroinflammatory response induced by social defeat.

It is well established that SD results in activation of microglia and increases inflammatory cytokine production (Calcia et al., 2016; Ferrer-Pérez et al., 2018; Finnell & Wood, 2016; Montagud-Romero et al., 2021; Rodríguez-Arias et al., 2017; Wohleb et al., 2011, 2012, 2015). Several studies have previously observed that animals susceptible to depressive-like symptoms induced by SD show an increased neuroinflammatory response (Ballestín et al., 2021; Nasca et al., 2019; Pfau et al., 2019; Reguilón et al., 2021a). Other studies have also observed increased levels of other pro-inflammatory cytokines such as MCP-1, IL-17 or IL-1β after exposure to social stress (Hodes et al., 2014; Stewart et al., 2015; Wood et al., 2015; Yang et al., 2021) and in signaling complexes that activate cytokines, e.g. NFkBp-p65, COX-2 and NLRP3 (Hodes et al., 2014; Montagud-Romero et al., 2021; Stewart et al., 2015; Yang et al., 2021). Also, increases in anti-inflammatory cytokines such as IL-4 and IL-10 have been observed in resilient animals (Hodes et al., 2014; Stewart et al., 2015). Using the same procedure employed in the present

study, we observed that defeated susceptible mice showed a long-term increase in the pro-inflammatory cytokine IL-6 in CPF, striatum and hippocampus (Ballestín et al., 2021; Reguilón et al., 2021a). In contrast, studies that have analyzed the chemokine CX3CL1 in defeated mice have reported different responses of this chemokine. After exposure to SD, reductions of CX3CL1 have been observed in striatum, hippocampus (Ballestín et al., 2021; Montagud-Romero et al., 2020; Reguilón et al., 2021a), hypothalamus and rostral cortex (Wohleb et al., 2013) in the C57BL/6 strain. In contrast, increases in CX3CL1 protein levels in striatum have been reported in another mice strain such as OF1 (Reguilón et al., 2020, 2021b).

The present results give support to the increased levels of the pro-inflammatory cytokine IL-6 in the striatum of susceptible mice subjected to SD (SD-S) compared to the CTRL and SD-R groups, although no differences in PFC were observed. Conversely, for the chemokine CX3CL1, in the PFC we observed significantly elevated levels of CX3CL1 also in the SD-S group, without significant differences in the striatum. This results contrast with previous results of our own laboratory in which SD mice showed exhibited no changes in this chemokine after exposure to cocaine-induced CPP either in resilient or susceptible mice (Ballestín et al., 2021). However, defeated mice presented lower levels of this chemokine in the PFC, with this decreased only evident in the striatum of susceptible mice (Reguilón et al., 2021a). Based on studies in humans and animal models in which increases in the levels of this chemokine have been observed in the striatum during alcohol exposure, dependence and withdrawal (Chen et al., 2011; Pascual et al., 2015), we hypothesized that opposite results observed in the present study could be due to the exposure to a 20% ethanol during the DID and SA procedures which could have altered CX3CL1-CX3CR1 signaling.

The novelty of the present study was that SI during adolescence prevented the increase in neuroinflammatory levels induced by social stress. In the PFC, decreased

levels of the pro-inflammatory cytokine IL-6 in all SI-exposed mice was observed compared to non-exposed mice. Equally, significant decreases of IL-6 were observed in the SI-SD-S group compared to the SD-S group in the striatum. In agreement with these results, lower neuroinflammatory response in environmentally enriched susceptible mice during adolescence has been reported (Reguilón et al., 2021a). With respect to the chemokine CX3CL1, we also observed a protective role of SI since there was a decreased level in the susceptible group exposed to SI (SI-SD-S) compared to the SD-S group in the PFC. In addition, significant differences were observed between the control groups, with increased CX3CL1 levels in the SI-CTRL group compared to the CTRL group. This increase may indicate that stress during adolescence sensitizes the immune system (Lo Iacono & Carola, 2018) and the reward pathway (Rodriguez-Arias et al., 2016). In contrast, no statistical differences were observed in the striatum. These data lead us to hypothesize the importance of the effects of social stress on the CX3CL1-CX3CR1 signaling pathway, if alcohol is producing a slight increase in CX3CL1 levels, SI exposure could be acting as protective having produced a decrease in protein levels prior to alcohol exposure. Undoubtedly, the effects of SD on the neuroinflammatory role of CX3CL1 should be further investigated.

The beneficial effects of SI on the decreased ethanol intake induced by SD, could be due by the decreased neuroinflammatory response. The present results have confirmed the relation between the resilient phenotype to depressive-like behavior and increased ethanol intake with a contained neuroinflammatory response. However, other mechanism must be also involved. The beneficial effects induced by SI may be due to a lower reactivity of the HPA axis to a subsequent stress experience and/or the development of a higher neuroplasticity or behavioral flexibility that characterizes the resilient phenotype (Hawley et al., 2010; Lambert et al., 2014; Lee et al., 2014). Taking into consideration the U-shaped function, mild and intense stressors would show an extreme HPA axis response, although moderate stress

would show a moderate axis response (Macrì et al., 2011). There are hardly any studies evaluating the HPA axis response in rodents subjected to SI during adolescence and the results are controversial. Some studies demonstrated reduced corticosterone response following SI training in adult animal (Brockhurst et al., 2015), although opposite results were obtained in adolescents (Mancini et al., 2021). Further studies need to be done to clarify the HHA response after SI experience.

5. Conclusion

The study of the mechanisms that explain the development of depression and vulnerability to drug addiction after exposure to social stress highlights the importance of developing preventive strategies that promote resilience to stress. The present study highlights the role of moderate SI in enhancing resilience to the effects of social stress. In summary, our results corroborate that SD produces depressive-like behaviors, enhances the reinforcing and motivational effects of ethanol, and induces a greater neuroinflammatory response in susceptible mice, in contrast to resistant animals. Our results indicated that training individuals in the face of exposure to stressful situations, adaptive responses to social stress can be developed. Adolescence is a critical developmental period for later mental illness, and an important period on which to focus intervention strategies. Further studies assessing the role of interventions during this period are needed to develop effective and individualized preventive strategies.

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Study 6

Resilience to social defeat stress in adolescent male mice

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(Annex 4)

Abstract

Adverse social experiences during adolescence are associated with the appearance of mental illness in adulthood. Social defeat (SD) is an ethologically valid murine model to study the consequences of social stress. In adolescent mice, SD induces depressive-like behaviors, increased anxiety and potentiates the reinforcing effects of cocaine and alcohol. However, not all mice exposed to SD will be susceptible to these effects. Adult mice resilient to the effects of SD show a consistent phenotype being resilient to depressive-like behaviors and to the increase in cocaine and alcohol consumption. The aim of the present study was to characterize the resilient phenotype to depressive-like behaviors and increase cocaine and ethanol rewarding effects of mice socially defeated during adolescence. To that end, adolescent mice were exposed to repeated SD, and 24 h after the last encounter, they underwent a social interaction test (SIT) in order to evaluate depressive-like behaviors. Cocaine-induced reward conditioning and ethanol intake was evaluated in two different sets of mice 3 weeks after the last SD using cocaine-induced conditioned place preference (CPP) and oral ethanol self-administration (SA). The neuroinflammation response was measured at the end of the experimental procedure by measuring striatal and cortical levels of IL-6 and CX3CL1. The results confirmed that a comparable percentage of adolescent mice develop resilience to depressive-like behaviors to that observed in adult mice. However, increased anxiety was more severe in resilient mice. Likewise, an increased preference for an ineffective dose of cocaine and an increased ethanol consumption was observed in resilient mice compared to controls. The increase in IL-6 and CX3CL1 was mainly observed in the striatum of susceptible mice compared to that of control mice. Our results confirm that, contrary to prior assumptions in adults, responses to SD stress are more complex and singular in adolescents, and caution should be taken for the correct interpretation and translation of those phenotypes.

Keywords: Social defeat; Susceptibility; Resilience; Neuroinflammation; Cocaine; Ethanol

Abbreviations:

BP: breaking point; CPP: conditioned place preference; DA: dopamine; ELISA: enzyme-linked immunosorbent assay; EPM: elevated plus maze; FR1: fixed ratio 1; FR3: fixed ratio 3; HPA: hypothalamic- pituitary- adrenal; PFC: prefrontal cortex; PND: postnatal day; Post-C: postconditioning; PR: progressive ratio; Pre-C: preconditioning; SA: self-administration; SD: social defeat; SIT: social interaction test; VTA: ventral tegmental area

1. Introduction

Adolescence is a critical period of development characterized, among other behaviors, by an increase in the time spent with peers, a change in the quality of social interaction and frequent appearance of feelings of rejection (Platt et al., 2013; Somerville et al., 2010). Adverse social experiences during adolescence have been strongly associated with the appearance of mental illness in adulthood. Many subjects who have suffered from abuse or have been abandoned by their parents during developmental periods are diagnosed in adulthood with a mental illness such as depression, anxiety or drug addiction (Ho & King, 2021). A recent meta-analysis reported that adverse childhood or adolescent experiences are highly associated with anxiety and depression, costing upwards of billions of dollars annually (Bellis et al., 2019). But the strongest association is found with problematic drug use and interpersonal and self-directed violence (Hughes et al., 2017). Specifically, clinical studies indicate that stressful adolescent experiences increase the risk for substance abuse (Tharp-Taylor et al., 2009; Topper et al., 2011). Due to the close relationship between the brain systems involved in the response to drugs and stress, environmental stressors can produce long-term changes in the brain reinforcement system, inducing the individual to use drugs (Rodríguez-Arias et al., 2013).

Animal models enable the study of the mechanisms through which environmental and psychosocial stressors induce later neuropsychiatric disorders. Social defeat (SD) is considered the most representative animal model to study the consequences of social stress (Hammels et al., 2015). SD is an ethologically valid murine model that induces long-term physiological and behavioral changes similar to those seen in depression and anxiety and can mimic the individual differences in the stress response observed in humans (Wang et al., 2021).

SD in adult mice potentiates the reinforcing effects of different drugs, producing an increased intake of cocaine and other psychostimulants on the self-administration

(SA) and conditioning of place preference (CPP) paradigms (Ballestín et al., 2021; Covington 3rd et al., 2008; Ferrer-Pérez et al., 2019; Giménez-Gómez et al., 2021; Quadros & Miczek, 2009; Montagud-Romero et al., 2016, 2020, 2021; Reguilón et al., 2017; Rodríguez-Arias et al., 2017). A significant increase in alcohol consumption after exposure to SD has also been reported (Montagud-Romero et al., 2021; Reguilón et al., 2020, 2021a, 2021b; Rodríguez-Arias et al., 2016). However, the effects of SD during adolescence on subsequent drug abuse or mental health have not been widely investigated.

Neural and behavioral development of rodents is thought to mirror stages of human development (Adriani & Laviola, 2004; Burke & Miczek, 2014). Like adolescent humans, adolescent rodents are highly social, to a greater extent than adult rodents (Do Couto et al., 2009; Yates et al., 2013). Defeated adolescent rats or mice show reduced social behavior, depressive-like behaviors, or increased anxiety similar to what is observed in defeated rodents in adulthood (Huang et al., 2013; Iñiguez et al., 2014; Shimizu et al., 2020). Similar to adults, increases in cocaine SA (Burke & Miczek, 2015), amphetamine-, cocaine-, and alcohol-induced conditioned reinforcement (Burke et al., 2011; Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017), and oral ethanol SA (Burke & Miczek, 2015; Rodríguez-Arias et al., 2016: Thompson et al., 2020) are observed in socially defeated adolescent rodents.

Nevertheless, not all subjects exposed to stress will develop depressive, anxiety or addictive behaviors. But as in humans, a subset of mice exposed to SD will be susceptible to these effects, developing important disorders such as social inhibition, anhedonia or depressive-like behaviors (Krishnan et al., 2007). However, some rodents will be resilient to these consequences, being able to adaptively cope with stress (Cathomas et al., 2019). We have recently reported that mice resilient to the effects of SD during adulthood show a consistent phenotype; that is, these mice are resilient to the depressive-like behaviors produced by SD, and are also resilient to the reinforcing effects of cocaine and alcohol (Ballestín et al., 2021; Giménez-

Gómez et al., 2021; Reguilón et al., 2021b). The response to SD seems to be much more complex in adolescent mice, and this also appears to be the case in the development of an adaptive response to stress. To date, only two studies have evaluated their resilience profile. Both studies reported that only a small proportion of the defeated adolescent mice (between 20 and 30%) were totally susceptible or totally resilient to certain effects of SD. However, these studies did not address the increased susceptibility to drug abuse (Alves-dos-Santos et al., 2020; Vassilev et al., 2021).

The aim of the present study was to characterize the resilient phenotype to depressive-like behaviors and the increased rewarding effects of cocaine and ethanol SA in socially defeated mice during adolescence. To that end, adolescent mice were exposed to repeated SD and, 24 h after the last encounter, underwent a social interaction test (SIT) in order to evaluate depressive-like behavior. Cocaine-induced reward conditioning and ethanol intake was evaluated in two different sets of mice 3 weeks after the last social defeat using cocaine-induced CPP and oral ethanol SA.

Recent studies suggest that the neuroinflammatory response may play an important role in the development of mental illness (Liu et al., 2020a; Soria et al., 2018), as the immune system also regulates the hypothalamic-pituitary-adrenal axis (HPA), thereby modulating the response to a stressful situation (Haroon et al., 2012). It is well known that social stress induces an activation of the immune system with short- and long-term increases in the levels of cytokines and chemokines (Ferle et al., 2020; Ferrer-Pérez et al., 2018; Jiang et al., 2020; Nozaki et al., 2020; Montagud-Romero et al., 2020; Reguilón et al., 2020, 2021a). These results have also been confirmed with SD in adolescent mice, which also showed an impairment of integrity in the blood-brain barrier and activation of the microglia (Rodríguez-Arias et al., 2017; Rodríguez-Arias et al., 2018; Zhu et al., 2019). We observed that this neuroinflammatory response is absent in socially defeated mice during adulthood that showed a resilient phenotype to depressive-like behaviors and increased cocaine

or ethanol intake (Ballestín et al., 2021; Reguilón et al., 2021b). Therefore, we will also characterize the neuroinflammatory response in defeated adolescent mice after cocaine or ethanol exposure, measuring the IL-6 and CX3CL1 level in the striatum and the prefrontal cortex (PFC).

In summary, we aim to characterize the behavioral response to the conditioned rewarding effects of cocaine and ethanol intake, as well as the neuroinflammatory response in mice with a resilient phenotype to the depressive-like effects induced by social defeat during adolescence.

2. Material and methods

2.1. Subjects

A total number of 77 adolescent male C57BL/6 J mice (Charles River, France) were used in this study. The experimental mice (PND 21) were housed in groups of five in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N = 20) and were individually housed in plastic cages (21 × 32 × 20 cm) for at least a month prior to the initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). All mice were housed in controlled laboratory conditions: constant temperature and humidity, and a reversed light schedule (lights off at 08:00 and on at 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (2019/VSC/PEA/0059 y 2019-VSC-PEA-122).

2.2. Drugs

For CPP, a dose of 1.5 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Spain) was employed and injected intraperitoneally (i.p.). This dose of cocaine was selected

based on previous CPP studies showing that doses below 3 mg/kg are sub-threshold (Arenas et al., 2014; Montagud-Romero et al., 2017; Vidal-Infer et al., 2012). Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drug. For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water.

2.3. Experimental Groups and Experimental Design

In this study, two different sets of mice were employed, all of which were exposed to the SD procedure or exploration from PND 27 to 36. 24 h after the last SD episode, on PND 37, all the mice performed the elevated plus maze (EPM) and the SIT to evaluate depressive-like behaviors. Subsequently, the first set of mice underwent the CPP procedure with 1.5 mg/kg of cocaine on PND 57, after 3 weeks of being undisturbed in their home cages. Mice were characterized as resilient or susceptible depending on their ratios in the SIT. Brain samples were taken at the end of the procedure (PND 65).

Likewise, after performing the SDs, EPM and SIT, the second set of mice initiated the 6% oral ethanol SA protocol on PND 57, 3 weeks after the last defeat, lasting approximately 28 days. During this paradigm, the mice proceeded through the phases of training (7 days), substitution of saccharin for ethanol (10 days), the FR1 (5 days), FR3 (5 days) and PR (1 day) schedules. At the end of this test, all the mice were sacrificed to obtain the brain samples for further analysis (PND 85).

The experimental design is depicted in Fig. 1.

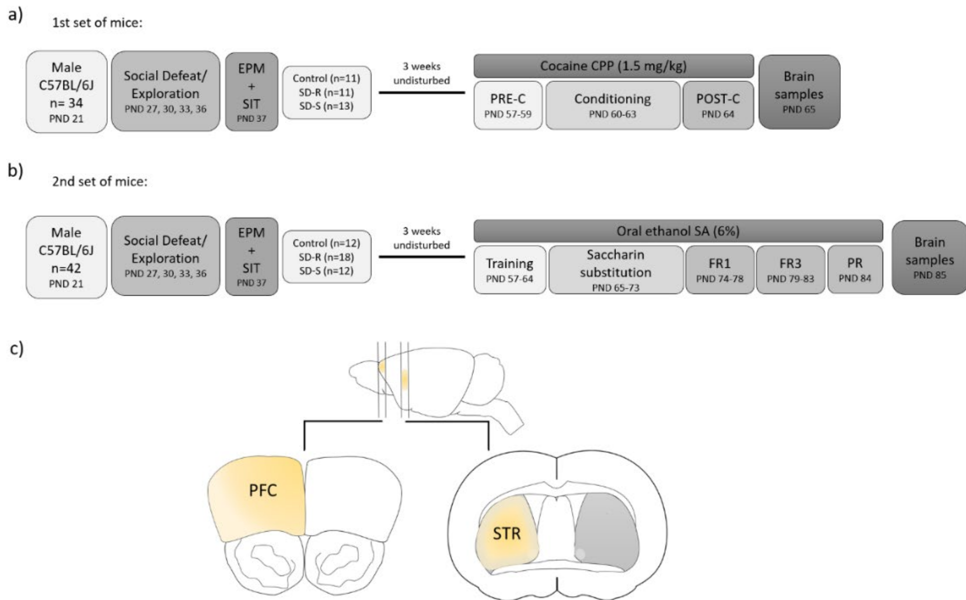

Fig. 1. Experimental design. Experimental protocol of the (a) first and (b) second sets of mice. (c) Diagram of the areas selected for immunoassay analysis. PFC = prefrontal cortex; STR = striatum.

2.4. Apparatus and procedures

2.4.1. Procedure of social defeat (SD)

Mice in the stress/defeated groups were exposed to 4 episodes of SD during adolescence, each lasting 25 min and consisting of three phases. The initial phase began by introducing the "intruder" (the experimental mice) into the home cage of the "resident" (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington 3rd & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two mice over a 5-min period. Finally, the wire

mesh was put back in the cage to separate the two mice once again for a further 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define a mouse as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). All agonistic encounters of each SD protocol were videotaped to confirm SD of the intruder mice and to ethologically analyze the threat and attack behaviors (duration and latency) of the resident mice. These behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice.

2.4.2. Elevated Plus Maze (EPM)

The EPM test was carried out essentially following the procedure described by Daza-Losada et al. (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, mice were habituated to the experimental room for 1 h prior to testing. At the beginning of each trial, the experimental mice were placed on the central platform facing an open arm and were allowed to explore for 5 min. The behavior displayed by the mice during the test was recorded by an automated tracking system (EthoVision XT 11, Noldus) that tracks the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms were measured to characterize the anxiolytic effects of the SD (Ferrer-Pérez et al., 2018; Rodríguez-Arias et al., 2016).

2.4.3. Social interaction test (SIT)

The social withdrawal ratio used was based on the social approach-avoidance test previously described by Berton et al. (2006). The test took place 24 h after the last SD during the dark cycle and in a different environment from the confrontation sessions. First, mice were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each mouse was placed in the center of a square arena (white Plexiglas open field, 30 cm each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Mice were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first session (object session), an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Although it can be argued that the probe mouse used in the social interaction test resembles the aggressor, and that this could foster social aversion, this is unlikely, since previous experiments demonstrate similar amounts of social investigation, irrespective of the strain used (i.e., C57BL/6; Berton et al., 2006). Before each session, the arena was cleaned with 5% alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Arena occupancy during object and social sessions was determined using the mice's horizontal position, controlled by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restrain cage. Corners were defined as two squares of similar areas on the opposite wall of the arena. Social withdrawal ratio is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction

zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, mice with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011).

2.4.4. Conditioned place preference (CPP)

For place conditioning, we employed eight identical Plexiglas boxes with two compartments of equal size (30.7 × 31.5 × 34.5 cm high) separated by a gray central area (13.8 × 31.5 × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the position of the mice and their crossings from one compartment to the other to be recorded. The equipment was controlled by three computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

Place conditioning, consisting of three phases, was carried out during the dark cycle following a procedure that is unbiased in terms of initial spontaneous preference (Manzanedo et al., 2001). During the first phase -preconditioning (Pre-C)- mice were allowed access to both compartments of the apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Mice showing a strong unconditioned aversion (<33% of session time; i.e. 250 s) or preference (>67% of the session time; i.e. 650 s) for any compartment were discarded from the rest of the study. The ANOVA showed no significant differences between the time spent in the drug-paired and vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, mice were conditioned with 1.5 mg/kg cocaine or saline. During this phase, half of the mice in each group received the drug or vehicle in one compartment, while the other half received it in the other compartment. An injection of physiological saline was

administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the mice received cocaine immediately prior to confinement in the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. The dose of cocaine used during the conditioning phase was a subthreshold dose (1.5 mg/kg, proven to be ineffective in controls) in order to evaluate increased sensitivity to the conditioned rewarding effects of cocaine. In the third phase -postconditioning (Post-C)-, which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice during a 900 s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug (conditioning score). If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

2.4.5. Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 36 µl of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6 s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, mice underwent an experiment carried out in three phases: training, saccharin substitution and 6% ethanol consumption.

2.4.5.1. Training phase (7 days)

Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and the food allotment was provided 1 h before the session to increase the motivation for active nose-poking. During the subsequent three days, water was provided ad libitum, except during the 1 h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1 h after the end of each daily session and water was available ad libitum to avoid ethanol consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 µl of 0.2% (w/v) saccharin reinforcement.

2.4.5.2. Saccharin substitution (10 days)

The saccharin concentration was gradually decreased as the ethanol concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% ethanol; 0.10% Sac −4% ethanol; 0.05% Sac −6% ethanol).

2.4.5.3. 6% ethanol consumption (11 days)

The aim of the last phase was to evaluate the number of active nose-poke responses, the 6% ethanol (w/v) intake and the motivation to drink. This phase began 38 days after the last SD. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, a fixed ratio 3 (FR3) (mice had to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a

progressive ratio (PR) session was completed to establish the breaking point (BP) for each mouse (the maximum number of nose-pokes each mouse is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1–2–3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the BP was calculated for each mouse as the maximum number of consecutive responses performed to achieve one reinforcement according to the previous scale. For example, if a mouse activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the BP value for this mouse would be 40. All the sessions lasted one hour, except the PR session, which lasted two hours.

2.4.6. Immunoassay analysis (ELISA)

Samples from the striatum and the PFC were obtained 24 h after cocaine CPP and oral ethanol SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed, and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Franklin & Paxinos, 2008, see Fig. 1c). The striatum and PFC were then kept in dry ice until storage at −80 °C. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

Some mice were discarded after measuring concentrations by ELISA due to a lack of signaling, and a few others were considered outliers.

2.5. Statistical analysis

Mice had been previously classified into resilient and susceptible groups based on their social withdrawal ratios. The social withdrawal ratio is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends on the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, mice with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups. The data of the time that the experimental mice and their aggressive opponents spent engaged in different behavioral categories during the SD episodes were compared by means of a mixed two-way ANOVA with one between-subject

variable Stress, with two levels (Resilient and Susceptible); and one within subject variable Days, with two levels (1st and 4th SD). To evaluate the CPP induced by 1.5 mg/kg of cocaine, the conditioning scores were analyzed with a one-way ANOVA with a between-subjects variable –Stress, with three levels (Control, SD-R and SD-S). To analyze the acquisition of ethanol SA, a two-way ANOVA was performed with one between-subjects variable –Stress with three levels (Control, SD-R and SD-S)– and a within-subjects variable –Days, with five levels of FR1 or FR3. The effects of SD and treatment on BP values and ethanol consumption during PR was analyzed by a two-way ANOVA, with one between-subjects variable –Stress. The data of the CX3CL1 and IL-6 levels, as well as the EPM, were analyzed using a one-way ANOVA with one between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible). For the data of the ethological analyses of SD and the EPM, all mice were analyzed together (1st and 2nd set) as the encounter, or the test occurred before the initiation of the cocaine CPP or the oral ethanol SA.

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. Statistical analyses were performed using SPSS Statistics (v.26; IBM, NY, USA) for behavioral data and GraphPad Prism (v8; GraphPad Software Inc., CA, USA) for graph design. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Classification between susceptible and resilient mice according to their social withdrawal ratios

In the first set of experimental mice (Fig. 2a), the Control group (n = 11) showed a mean social interaction ratio >1. Of 24 SD mice, 54% had interaction ratio <1 and 46% showed a ratio equal to or higher than 1. We classified mice with an interaction ratio <1 as SD-S mice (n = 13) and those with an interaction ratio greater than or equal to one as SD-R mice (n = 11).

In the second set of experimental mice (Fig. 2b), the Control group (n = 12) showed a mean ratio higher than 1. In the SD group of mice (n = 30), 40% showed a ratio under 1, which classifies them as susceptible (SD-S) mice (n = 12), and the remaining 60% showed a ratio equal to or higher than 1, which classifies them as resilient (SD-R) mice (n = 18).

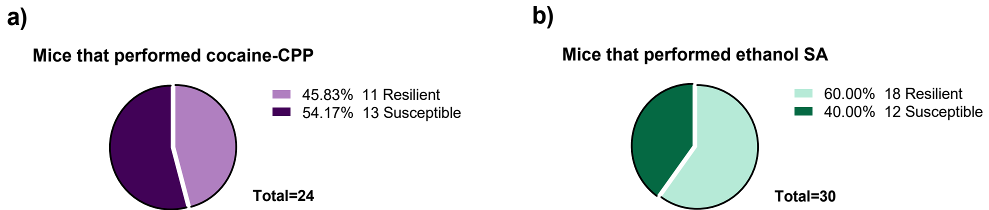

Fig. 2. Percentages of resilient and susceptible mice among groups of mice defeated during adolescence in the two experimental sets. The pie chart represents the percentage of resilient vs susceptible mice after social withdrawal ratio evaluation in the SIT in a) defeated mice that performed cocaine-CPP and b) defeated mice that performed ethanol SA paradigm.

3.2. All defeated adolescent mice increased passive-reactive coping during the 4th social defeat

The ANOVA for the time employed in Avoidance/Flee or Submissive/Defensive behaviors (Table 1) by defeated mice divided into resilient or susceptible according to their SIT scores showed an effect of the variable Days [$F(1,52) = 16.5$; $p < 0.001$] and [$F(1,52) = 9.1$; $p < 0.001$]. Likewise, the ANOVA for the latency to show Avoidance/Flee or Submissive/Defensive behaviors for the first time revealed an effect of the variable Days [$F(1,52) = 16.2$; $p < 0.001$] and [$F(1,52) = 25.9$; $p < 0.001$]. Defeated adolescent mice, classified as either resilient or susceptible, increased the time spent in these behaviors and were quicker to show them during the 4th social defeat ($p < 0.01$ for time spent in Submissive/Defensive behaviors and

p < 0.001 for the rest of comparisons). The lack of differences depending on the SIT means that susceptible and resilient mice cope similarly with social defeat stress.

The ANOVAs for the time employed in Attack or Threat behaviors (Table 1) by resident mice showed an effect of the variable Days [F (1,52) = 16.9; p < 0.001] and [F (1,52) = 8.1; p < 0.01]. Resident mice decreased the time spent in these behaviors during the 4th SD compared to the 1st SD (p < 0.001 for time spent in Attack behavior and p < 0.01 for the time spent in Threat behavior). The ANOVAs for the latency to perform the first Attack revealed an effect of the interaction Days x Group [F (1,52) = 4.9; p < 0.05] and an effect of the variable Days [F (1,52) = 6.7; p < 0.05] for the latency to perform the first Threat behavior. Resident mice threatened faster in the 4th SD in comparison to the 1st SD in all groups (p < 0.05). However, resident mice attacked faster during the 4th SD only when attacking the SD-S group (p < 0.001).

Table 1. Coping behavior of the intruder mice during SD.

			SD-R		SD-S	
			1st SD	**4th SD**	**1st SD**	**4th SD**
Intruder mice	**Submissive/ Deffensive**	**Time (s)**	35 ± 4	50 ± 4 **	39 ± 4	52 ± 5 **
		Latency (s)	30 ± 6	11 ± 2 ***	40 ± 8	5 ± 1 ***
	Avoidance/ Flee	**Time (s)**	74 ± 7	116 ± 12 ***	72 ± 8	101 ± 15 ***
		Latency (s)	17 ± 3	7 ± 2 ***	26 ± 7	4 ± 1 ***
Resident mice	**Attack**	**Time (s)**	61 ± 5	38 ± 4 ***	60 ± 6	44 ± 4 ***
		Latency (s)	21 ± 3	19 ± 5	29 ± 6	9 ± 2 ###
	Threat	**Time (s)**	31 ± 5	17 ± 2 **	30 ± 5	26 ± 3 **
		Latency (s)	23 ± 5	17 ± 5 *	33 ± 10	10 ± 2 *

Results are presented as mean values ±SEM. Mice were divided into resilient (n = 29) and susceptible (n = 25) depending on their SIT scores. Bonferroni post-hoc test *p < 0.05, **p

< 0.01, ***p < 0.001 significant difference compared to the 1st SD. ###p < 0.001 significant difference compared to the 1st SD in SD-S group.

3.3. Social defeat during adolescence induced anxiogenic effects in resilient mice

One outlier in time spent in the closed arms was removed from the Control group, along with two outliers in time spent in the open arms and in percentage of time in the open arms, and one outlier in the number of entries to the open arms, in total entries and in percentage of entries to the open arms in the SD-S group.

The data of the EPM test are presented in Fig. 3. The ANOVA of the time spent in closed arms [$F_{(2,72)} = 4.5$; $p = 0.014$]; time spent in the open arms [$F_{(2,72)} = 3.9$; $p = 0.023$]; percentage of time spent in the open arms [$F_{(2,72)} = 3.5$; $p = 0.034$]; number of entries into the open arms [(2,73) = 3.6; $p = 0.031$], and percentage of entries into the open arms [(2,73) = 4.4; $p = 0.015$] revealed a significant effect of the variable Stress. Post-hoc analyses showed that resilient defeated mice according to their SIT scores spent more time in the closed arms, and less time and a lower percentage of time in the open arms than control non-stressed mice ($p < 0.05$ in all cases). Moreover, a lower number and percentage of entries into the open arms was registered in resilient defeated mice ($p < 0.05$ in all cases) with respect to the control group.

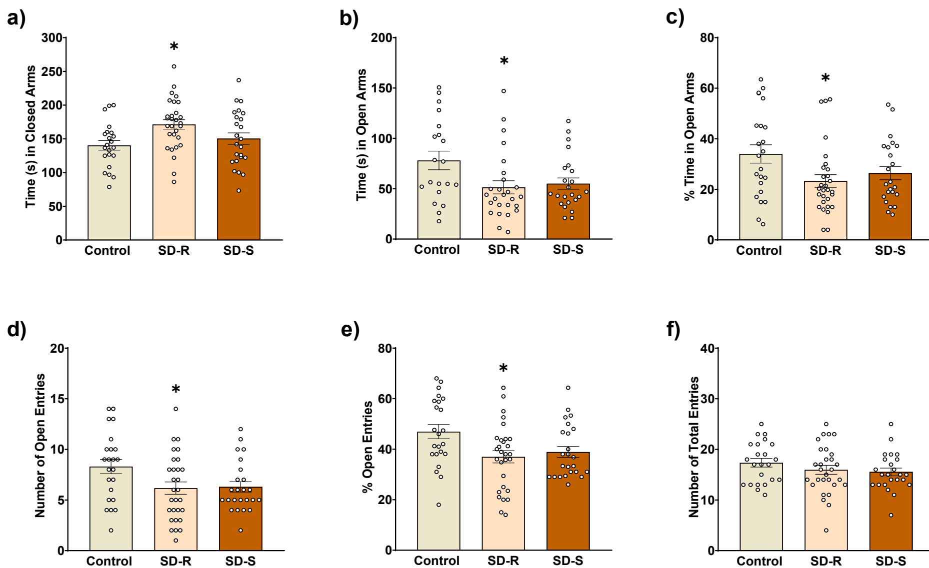

Fig. 3. Long term effects of SD on anxiety-like behavior. Bars represent the mean (± SEM) of (a) time in closed arms in seconds, (b) time in open arms in seconds, (c) percentage of time in open arms, (d) number of open entries, (e) percentage of open entries and (f) number of total entries. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

3.4. Mice considered resilient according to their SIT scores developed a preference for cocaine-induced CPP

The ANOVA for the time spent in the drug-paired compartment (Fig. 4a) showed a significant effect of the variable Days [$F(1, 37) = 5.2$, $p = 0.030$], and the interaction Days x Stress [$F(1,31) = 3.3$, $p = 0.05$]. Likewise, the ANOVA of the Conditioning Score (Fig. 4b) showed an effect of the variable Stress [$F(2,31) = 4.7$; $p < 0.016$]. Only resilient mice according to their SIT scores developed a preference for this cocaine dose and a significant increase in the time spent in the drug-paired compartment during the Post-C test was observed (p 〈0,01). Consequently, higher conditioning scores were observed in the resilient mice than control mice ($p < 0.01$).

In addition, the analysis of the differences between the drug-paired and vehicle-paired compartments during the Pre-C and Post-C phases can be found in Table A of the supplementary material.

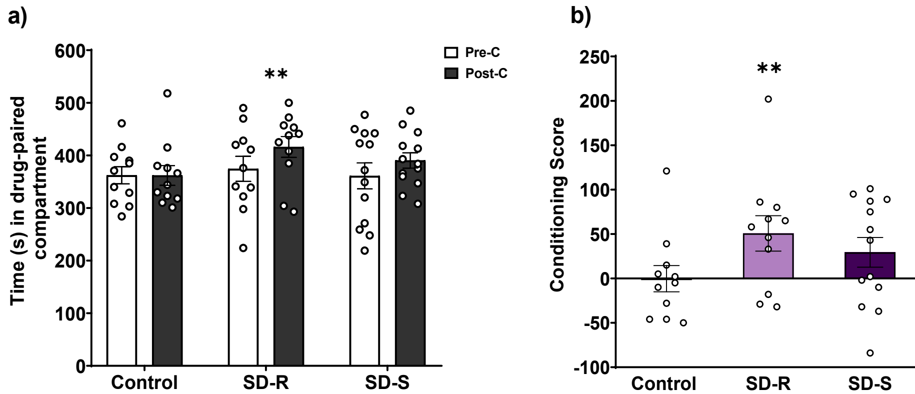

Fig. 4. Resilient mice showed higher preference in cocaine-induced CPP than susceptible mice. Effect of adolescent SD on cocaine-induced CPP. Mice were divided into Control (n = 11); Resilient (n = 11) and Susceptible (n = 13). Defeated mice were characterized as resilient or susceptible depending on their SIT scores. a) The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (Presingle bondC) (white bars) and after conditioning sessions in the post-conditioning test (Post-C) (gray bars), during which CPP was induced with 1 mg/kg of cocaine. b) The bars represent the conditioning score (difference in seconds between the time spent in the drug-paired compartment after the conditioning sessions and that spent in the same compartment during Presingle bondC). **$p < 0.01$ significant difference in the time spent in the drug-paired compartment vs Pre-C session or with respect to the control group.

3.5. Increased neuroinflammatory response observed following cocaine-induced CPP in susceptible mice compared to control mice

The number of samples in each group was 10 for IL-6 and between 11 and 13 for CX3CL1. For IL6 determination, we lost 2 samples in the striatum of the control

group (outliers), and another 2 in the striatum of the SD-R group (one outlier and one due to lack of signaling).

The ANOVA for the Il-6 levels in the striatum (Fig. 5a) showed a significant effect of the variable Stress [$F(2,26) = 3.9$; $p < 0.034$]. A higher concentration of Il-6 was observed in the striatum of susceptible mice according to their SIT scores compared to non-stressed control mice ($p < 0.05$). No differences were observed in the cortex (Fig. 5b).

With respect to fractalkine or CX3CL1 levels, although there were no differences in the striatum (Fig. 5c), a higher concentration was observed in the PFC of both susceptible and resistant mice [$F(2,35) = 4.5$; $p < 0.019$] compared to non-stressed controls ($p < 0.05$ in both cases) (Fig. 5d).

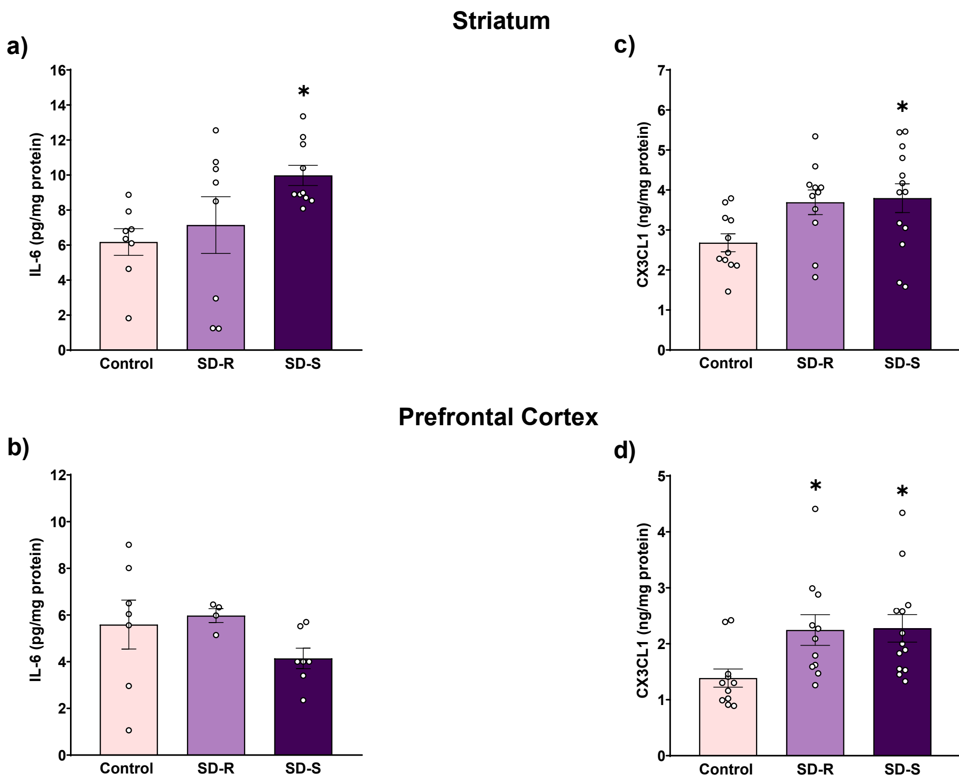

Fig. 5. Susceptible mice showed higher neuroinflammatory markers after cocaine-induced CPP than resilient mice. Effect of repeated SD on striatal and cortical levels of IL-6 and CX3CL1. Bars represent the mean (± SEM) of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6. Similarly, bars represent the mean (± SEM) of the striatal (b) and cortical (d) levels (in ng/mg) of the pro-inflammatory chemokine CX3CL1 and the vertical lines ± SEM. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

3.6. Resilient mice showed higher ethanol intake than control mice

No differences were found in the active responses or between the different groups during the training or substitution phases, demonstrating that SD did not induce any learning deficits. No differences were found in the body weight of the mice during the FR1, FR3 and PR schedules. Analyses of the acquisition and substitution phases of SA and body weights during the FR1, FR3 and PR schedules can be found on the supplementary material.

The ANOVA for the number of active responses during FR1 schedule (Fig. 6a) did not reveal any significant effects, although there was a tendency toward a Stress effect [$F(2,39) = 3.1$; $p = 0.058$). Resilient mice tended to perform more active responses than controls ($p < 0.058$). With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Stress [$F(2,39) = 4.5$; $p = 0.018$) (Fig. 6b). The post-hoc comparison showed that the resilient mice consumed ethanol at higher rates than the control ($p < 0.05$) and susceptible mice ($p < 0.01$).

During the FR3 schedule, the ANOVA of the number of active responses (Fig. 6a), the ANOVA revealed a significant effect of the variable Days [$F(4,156) = 4.4$; $p < 0.002$] and Stress [$F(2,39) = 6.3$; $p < 0.004$]. All mice made significantly more active responses on days 9 and 10 than on day 6 ($p < 0.01$ for day 9, and $p < 0,05$ for day 10). Moreover, resilient mice performed more active responses during the FR3 than non-stressed controls ($p < 0.01$). With respect to the ethanol consumption, the ANOVA revealed a significant effect of the variable Days [$F(4,156) = 16.4$; $p <$

0.001] and Stress [F(2,39) = 6.3; p < 0.004] (Fig. 6b). All mice consumed significantly more ethanol on days 7, 8, 9 and 10 than on day 6 (p < 0.001 in all cases). Moreover, resilient mice consumed more ethanol during the FR3 than non-stressed controls (p < 0.01).

During the PR, the ANOVA for the BP values of ethanol SA revealed a significant effect of the variable Stress [F(2,39) = 4.9; p < 0.012] (Fig. 6c). The post-hoc comparison showed that resilient mice achieved higher BP values than the control group (p < 0.01). The ANOVA for ethanol consumption during PR did not reveal a significant effect of the variable Stress (Fig. 6d).

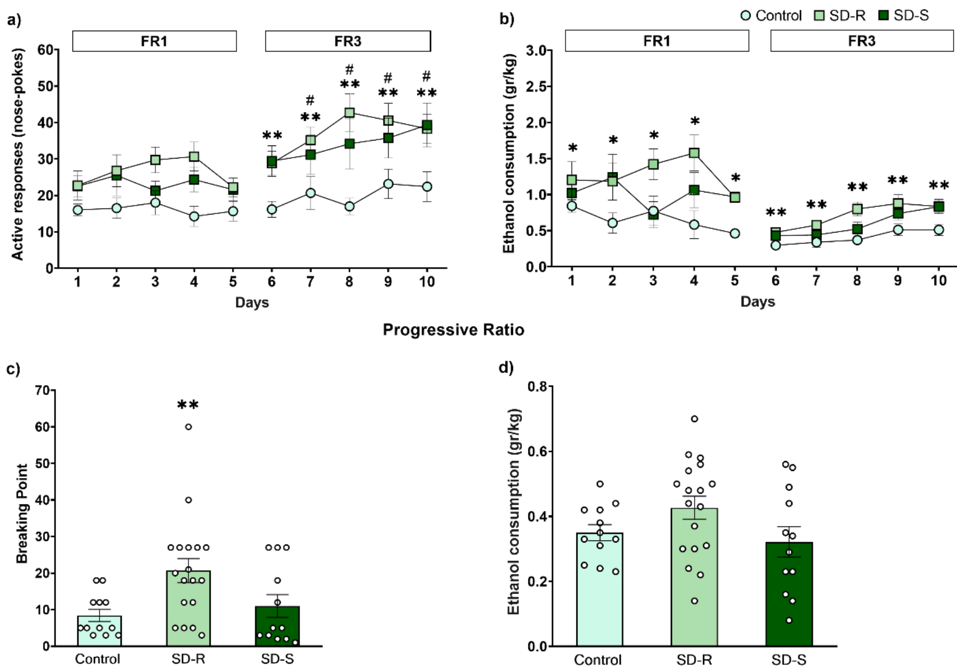

Fig. 6. Resilient mice showed higher ethanol intake than susceptible mice. Mice were divided into Control (*n* = 12); Resilient (*n* = 18) and Susceptible (n = 12). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses; (b) the volume of 6% ethanol consumption during FR1 and FR3; (c) the BP values; and (d) the volume of 6%

ethanol consumption during PR. *$p < 0.05$, **$p < 0.01$ significant difference with respect to controls; #$p < 0.05$ significant difference with respect to day 6.

3.7. Increased neuroinflammatory response observed in susceptible mice compared to control mice following oral ethanol self-administration

The number of samples in each group was between 11 and 13 for IL-6 and between 12 and 14 for CX3CL1. For CX3CL1 determination, we lost 3 samples in the SD-R group (three outliers).

The ANOVA for the Il-6 levels in the striatum (Fig. 7a) showed a significant effect of the variable Stress [$F(2,36) = 3.9$; $p < 0.023$]. A higher concentration of Il-6 was observed in the striatum of susceptible mice according to their SIT scores compared to the non-stressed control group ($p < 0.05$). No differences were observed in the PFC (Fig. 7b).

With respect to fractalkine or CX3CL1 levels in the striatum (Fig. 7c) the ANOVA showed a significant effect of the variable Stress [$F(2,33) = 3.8$; $p < 0.03$], as significantly elevated levels were observed in the susceptible mice compared to the control group ($p < 0.03$). No differences were observed in the PFC (Fig. 7d).

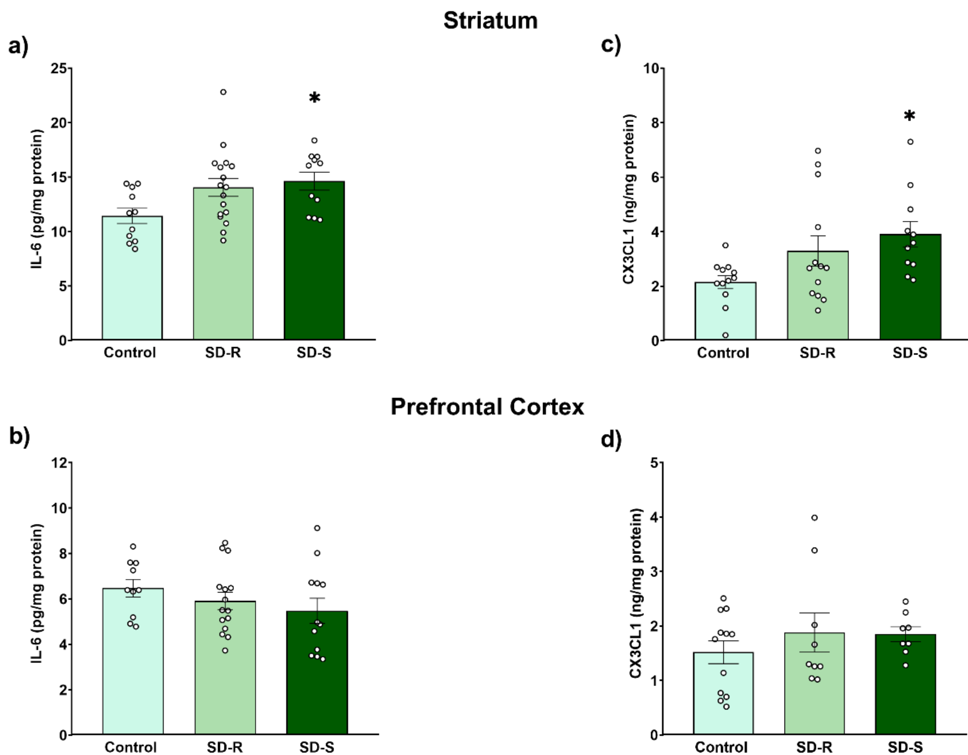

Fig. 7. Susceptible mice showed higher neuroinflammatory markers after oral ethanol self-administration than resilient mice. Bars represent the mean (± SEM) of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6. Similarly, bars represent the mean (± SEM) of the striatal (b) and cortical (d) levels (in ng/mg) of the pro-inflammatory chemokine CX3CL1 and the vertical lines ± SEM. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

4. Discussion

A recent meta-analysis reported that 62.5% of individuals start to show signs of mental disorders by the age of 25, with a peak at 14.5 years of age (Solmi et al., 2021). Although there is strong evidence linking bullying and later mental illness (McKay et al., 2021), only few adolescents suffering from these traumatic events will develop psychiatric disorders (Dumont & Provost, 1999; Aarestad et al., 2021).

Studies suggest that a correct reaction of the body is crucial for an adaptive response to the environment and to avoid stress-related deficits (Cathomas et al., 2019; Dutcher & Creswell, 2018). We already know that a percentage of adult rodents exposed to SD will show a resilient phenotype to social avoidance and to the increase in the rewarding effects of cocaine and ethanol intake (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). However, few studies have been performed to know whether stressed adolescent mice will show a consistent resilient phenotype, as adult mice do. The fact that the developmental process of resilience seems to strengthen over time, together with the increased salient value of social interactions in adolescent mice, indicates that the study of the resilient phenotype in adolescent defeated rodents is highly needed (Sheth et al., 2017; Malhi et al., 2019).

Our results showed that mice defeated during adolescence showed marked differences in their resilient response in comparison with the experience of SD during adulthood. Resilience to the detrimental effects of experiencing SD during adolescence did not develop as a unique phenotype. Mice resilient to depressive-like behavior showed an increased anxiogenic behavior, a higher response to cocaine and higher ethanol intake compared to the control group. However, the increased neuroinflammatory response was present mainly in the mice susceptible to depressive-like behaviors compared to the control group, despite showing a normal response to cocaine and ethanol.

4.1. Adolescent defeated mice showed behavioral flexibility coping with stress

We know that passive coping strategies in response to social stress are associated with more pronounced physiological effects and psychopathology (Hawley et al., 2010; Russo et al., 2012; Wood & Bhatnagar, 2015). Submissive and immobile behavior are considered passive-reactive coping strategies. Meanwhile, active

coping is characterized by longer latency to display the defeat posture, fight-back or active escape (Koolhaas et al., 2007; Wood et al., 2010).

We have previously reported that adult mice resilient to the increase in cocaine reward display fewer flee/avoidance and submissive/defensive behaviors during SD than those categorized as susceptible according to their SIT scores (Ballestín et al., 2021; Ródenas-González et al., 2021). In contrast with these results, there were no differences between resilient and susceptible adolescent mice in the way of coping with SD. All defeated adolescent mice showed an increase in the time spent in defensive or flee behaviors in the fourth SD and presented shorter latencies to show these behaviors. The increased time in these behaviors indicates behavioral flexibility to the inescapable SD experience and is characteristically observed only in resilient adult mice. Behavioral flexibility has been associated with emotional resilience, less reactivity of the HPA axis, and increased neuroplasticity (Hawley et al., 2010; Lambert et al., 2014). Therefore, the adolescent response to SD indicates an adequate adaptation to stress.

Despite this adaptive response to SD, the percentage of resilient and susceptible adolescent mice classified according to the SIT, which evaluates depressive-like behaviors, is similar to that observed in adult defeated mice (Giménez-Gómez et al., 2021), with 53% of adolescent defeated mice showing a resilient phenotype to depressive-like behaviors. Albeit with slightly methodological differences, this percentage was also observed in the study of Alves-dos-Santos et al. (2020) and Vassilev et al. (2021) with adolescent defeated mice.

One limitation of the present study is the fact that resilient and susceptible mice were housed together in the same cage throughout the entire procedure. Mice were housed in groups of 4 when they arrived at the laboratory on PND 21 and resilient or susceptible phenotypes to depressive-like behaviors were evaluated 24 h after the last SD on PND 37. Housing resilient and susceptible mice together at that moment

would have implied a deeply stressful hierarchical reorganization in each cage, which could affect the behavioral and biochemical results. Therefore, housing resilient and susceptible mice in the same cage must be taken into consideration as a potential variable that could affect the results obtained. Another variable to take into account is the fact that injuries derived from confrontations within the home cage could affect neuroinflammatory markers. However, we find this possibility very unlikely, as the condition of the mice was monitored daily and injuries among adolescent C57BL/6 J strain mice were not observed.

4.2. Anxiogenic response in resilient mice

It has been extensively established that SD induces an acute anxiogenic response (Albrechet-Souza et al., 2017; Ferrer-Pérez et al., 2019; Macedo et al., 2018), an effect that is lacking in resilient mice. We have previously observed that adult mice defeated during adulthood that present a resilient phenotype to depressive-like behaviors and the rewarding effects of cocaine did not present this anxiety increase. In addition, all the environmental or pharmacological treatments that increase the percentage of resilient mice to SD also increase the percentage of mice that did not experience an anxiogenic response (Giménez-Gómez et al., 2021). However, when the SD took place during adolescence, the results are controversial and the experimental protocol used to induce social stress and the time proximity between behavioral tests and stress play a key role in the response observed. Several reports confirmed that after 3 weeks of the last SD there were no observable effects in the EPM in defeated adolescent mice (Rodríguez-Arias et al., 2016; Watt et al., 2009). Alves-dos-Santos et al. (2020) did not observe anxiety-like behaviors in the EPM test when compared to control mice. In that case, the EPM took place in the last days of 10 sessions of chronic social defeat stress and mice were isolated during the entire procedure, which could affect the response. However, in agreement with Iñiguez et al. (2014), we observed that all defeated mice showed an increase in anxiogenic

behaviors 24 h after the last encounter, but only resilient mice to depressive-like behavior spent less time and percentage of time in the open arms. Different results were observed by Vassilev et al. (2021) with no increased anxiety observed in susceptible mice, but surprisingly, in resistant mice an anxiolytic response was observed with an increase in the time spent in the open arms, which the authors suggest may be due to higher propensity for risk-taking-like behaviors in resilient adolescent mice.

Therefore, our results indicate that, differently from the adult response to SD, adolescent mice developed a partial resilient response to anxiety and this was not associated with depressive-like behaviors.

4.3. Increased response to cocaine and ethanol in resilient mice compared to the control group

As we have previously reported for adult mice, a subset of defeated adolescent mice developed a preference for cocaine (Ballestín et al., 2021; Giménez-Gómez et al., 2021). However, different from adult defeated mice, adolescent mice resilient to depressive-like behaviors according to their SIT scores showed a preference for this dose of cocaine. On the other hand, susceptible mice showing social avoidance did not develop a preference for cocaine. However, we should note that a significant preference for the drug-paired compartment compared to the vehicle-paired compartment was observed during the post-conditioning phase in all defeated mice (see Table A in the Supplementary Material). Although SD-S mice did not develop a preference or an increase in the conditioning score, social defeat seems to exert some effect on cocaine preference compared to vehicle administration.

In line with the results observed with cocaine, resilient adolescent mice according to their SIT scores showed higher ethanol intake than control mice. Once again, defeated adolescent mice behave differently from adults. Resilient adult mice according to their SIT scores showed a complete phenotype with no social avoidance

and drinking a similar amount of ethanol as non-stressed control mice. On the other hand, defeated adult mice susceptible to social avoidance presented a significantly higher ethanol intake (Reguilón et al., 2021b).

In our defeated adolescent mice, the analysis of the two phenotypes (depressive-like behaviors and response to cocaine or ethanol) showed that, among mice resilient to depressive-like behaviors (with SIT scores superior to 1), 45% did not develop a preference for cocaine (see Table 2). Based on previous studies with this strain of mice, we considered that an increase similar or superior to 60 s was the minimum increase necessary to develop a preference (Ballestín et al., 2021; Giménez-Gómez et al., 2021). Therefore, only 21% of the defeated mice showed resilience to both phenotypes. Similar results were also obtained in the 2nd experiment, with only 33% of resilient mice depending on the SIT drinking ethanol similarly to control mice. In that case, mice that drank ethanol more than two standard deviations from the control mean were considered susceptible. As in the 1st experiment, 20% of the defeated mice showed a complete resilient phenotype. Therefore, there is an inconsistent development of resilience between depressive-like behaviors and response to drug reward.

4.4. Increased neuroinflammatory response in susceptible mice compared to the control group

Psychological stress induces a series of neuroimmune reactions involving a bidirectional brain-immune signaling that affects mood and behavior (Wohleb et al., 2015). Long-term increments in proinflammatory cytokines such as IL-6 levels after repeated SD have been described in several mice brain areas (Ferrer-Pérez et al., 2018; Montagud-Romero et al., 2020, 2021; Reguilón et al., 2021b). In adult mice, resilience to depressive-like behaviors and the increase in cocaine or ethanol reward are associated with a minor neuroinflammatory response. In susceptible mice, an increase in IL-6 levels was observed compared to those of controls or resistant mice

shortly after SD or after cocaine or oral ethanol SA (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). These results have also been confirmed in adolescent defeated mice. Although no increased response to cocaine or ethanol was observed in the susceptible mice, an increase in IL-6 levels was observed in the striatum after cocaine-induced CPP and oral ethanol SA, compared to the control group.

With respect to the chemokines response, SD induced changes in CX3CL1 or fractalkine, which seems to depend on the mouse strain used, probably due to their different sensitivity to social stress. Although SD induces increases of striatal CX3CL1 levels in OF1 mice (which are highly territorial) (Reguilón et al., 2020, 2021a), in C57BL/6 mice the opposite result was found. Both resilient and susceptible mice, decreases in striatal fractalkine levels were observed immediately after the last SD and even after cocaine-induced CPP (Ballestín et al., 2021). Moreover, after oral ethanol SA, decreases were only observed in susceptible mice (Reguilón et al., 2021b). Surprisingly, compared with the control group, increased levels of CX3CL1 were observed in the striatum of adolescent susceptible C57BL/6 mice following exposure to cocaine or ethanol, and cortical levels were increased in all adolescent defeated mice only following exposure to cocaine. Only few studies have evaluated this chemokine in adolescent mice, but a recent study by Liu et al. (2020b) found increased CX3CL1 expression in the hippocampus of adolescent mice exposed to nicotine during gestation and lactation.

Exposure to social stress during early adolescence produced a permanent alteration of microglia morphology and the induction of an inflammatory episode in the ventral tegmental area (VTA) (Lo Iacono et al., 2018). This inflammatory episode altered the functionality of dopaminergic neurotransmission in the VTA following exposure to a cocaine-CPP in adulthood. The authors of this study concluded that social stress during early life sensitizes the reward pathway and the immune response. In line with

this, the inflammatory responses observed in our study may be potentiated by the profound alteration of the immune system produced by social defeat in combination with subsequent exposure to substances of abuse.

4.5. Characteristic development of resilience to SD in adolescent mice

In these experiments, we demonstrated that experiencing SD during adolescence presents specific characteristics. In agreement with the few studies performed in this area, defeated adolescent mice did not develop a general resilient/susceptible phenotype. There is no correlation between the resilience to social avoidance and the increased response to cocaine and ethanol or the neuroinflammatory response. Although, differently from adults, all adolescent mice presented an adaptive coping mechanism with stress, the percentage of resilient/susceptible mice after SD is comparable to that observed in adult mice. Increased anxiogenic behavior, preference for the cocaine-paired compartment or ethanol intake were observed in mice resilient to the development of social avoidance. However, resilience to social avoidance correlated with a minor neuroinflammatory response. These results indicate that the age of exposure to SD affects the development of resilience.

In line with our results, Alves-dos-Santos et al. (2020) observed that defeated adolescents resilient to anhedonia or social avoidance were the most affected mice in terms of both endocrine/physiological outcomes (body weight gain and corticosterone response). Likewise, Vassilev et al. (2021) observed that, in adolescence, SD produces inhibitory control impairment independently from social avoidance. As with ours, all these studies have been performed only in males, which is an important limitation of the present investigation. Marked sex differences in stress responses have been reported in adult rodents and humans (for reviews, see Hodes & Epperson, 2019; Wellman et al., 2018), but limited data in female rodents during adolescence are available.

Resilience should be considered an active process, which affects both passive and active strategies, in order to achieve the highest adaptation to stress (Russo et al., 2012). Responses on each particular system may develop differently after exposure to stress (Smith, 2019). Our results suggest that SD during adolescence leads to an addiction-prone phenotype in some mice, which manifests itself as resilient during the SIT and presents a normalized neuroinflammatory response.

The response to drugs of abuse is based on their rewarding properties, which depend on the function of the mesocorticolimbic dopaminergic system. Vassilev et al. (2021) observed that SD during adolescence, but not in adulthood, dysregulates the Netrin-1/DCC pathway in the VTA and the nucleus accumbens, which induced changes in dopamine (DA) connectivity in the PFC. These authors observed that, although a reduction in VTA DCC expression was observed in all defeated mice, ectopic growth of mesolimbic DA axons was observed into the medial PFC of resistant mice. This specific adaptation on the dopaminergic system could be related to the increased response to cocaine and ethanol observed in resilient mice.

5. Conclusions

Adolescence is a critical developmental period for later mental illness, and an important period in which to focus intervention strategies. More studies are needed in order to fully evaluate the relationship between bullying and substance use disorders. A recent study showed a bidirectional correlation indicating that individuals who engaged in substance use were more likely to perpetrate cyber aggression than those who did not, a result that suggests a strong relationship between substance use and bullying (Crane et al., 2021).

Our findings illustrate that, contrary to prior assumptions in adults, SD stress responses are more complex and singular in adolescents, and caution should be taken for the correct interpretation and translation of those phenotypes. The Social Interaction Test, considered a depressive-like phenotype, is currently used to classify

mice into resilient or susceptible in order to study the neurobiology and molecular aspects of social stress (e.g. Russo et al., 2012). In adolescents, we should not assume that resilience to one phenotype equally develops for others, highlighting the concept of resilience as an active process affected by the person's age at the moment of the stress experience.

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Study 7

Morphological changes induced by social stress and ethanol intake in male mice

Reguilón, M.D.

Pilot study

1. Introduction

The present study was carried out during my research stay at the Neurotoxicity, Neuroprotection and Neurorepair laboratory of the Institute of Cell Biology and Neuroscience "Prof. E. De Robertis" (IBCN-CONICET) at the Faculty of Medicine of the University of Buenos Aires (UBA), Argentina. In the following I will present some of the data obtained that have already been fully analyzed.

In the scientific literature we can find several studies evaluating the neural substrate of the phenomenon of resilience. Synaptic plasticity may be the fundamental mechanism to respond adaptively to adverse changes in the environment (Montes-Rodriguez & Urteaga-Urias, 2018). The effects of social defeat (SD) on synaptic plasticity have not been extensively studied. Recently, changes in inhibitory synapses have been observed, with reduced inhibitory synaptic transmission within the nucleus accumbens (NAc) core being critical in the stress response (Heshmati et al., 2020). However, there are few reports on the changes in neuronal and synaptic morphology of those induced by some form of social stress. For example, in the study of changes in dendritic extensions, low levels of microtubule-associated protein 2 (MAP-2) have been observed in humans in hippocampal formations of patients with major depression (Soetanto et al., 2010). In preclinical models of chronic SD stress, chronic unpredictable mild stress, and maternal separation, stressed rodents were observed to undergo decreased MAP-2 gene expression in the hippocampus and cerebral cortex (Abdel-Rahman et al., 2004; García-Gutiérrez et al., 2016; Martin et al., 2017; Yang et al., 2015), this dendritic and synaptic alteration could lead to cognitive alterations (Abdel-Rahman et al., 2004; Martin et al., 2017), influence the development of depressive-like behaviors (García-Gutiérrez et al., 2016; Yang et al., 2015) and vulnerability to ethanol consumption during adolescence (García-Gutiérrez et al., 2016). Likewise, in the prenatal stress model in rats, a decrease in MAP-2 immunostaining levels was observed in cortex, striatum and hippocampus, this decrease in dendritic arborization could lead to a

reduction in neuronal processes and a decrease in the number of synapses (Barros et al., 2006). In relation to the axonal cytoskeleton, there are few scientific reports showing that social stress causes a decrease in the expression of neuronal 200 kDa filaments (NF200) in mice subjected to maternal separation (García-Gutiérrez et al., 2016). NF200 plays an important role in the stabilization and maturation of pre-existing connections, a dysregulation of its expression can derive in an impairment of synaptic connections (García-Gutiérrez et al., 2016).

In relation to the astrocytes cytoskeleton, conflicting results have been described in the scientific literature. It is known that astrocytes are particularly sensitive to glucocorticoids and the release of pro-inflammatory markers leads in turn to astrogliosis, causing structural and functional changes in the brain (Calcia et al., 2016). Depending on the structure analyzed and the type of social stress used, decreases in glial fibrillary acidic protein (GFAP) expression derived from exposure to chronic and repeated SD have been observed in structures such as the hippocampus, NAc and the prefrontal cortex (PFC; Araya-Callís et al., 2012; Rappeneau et al., 2016; Rodríguez-Arias et al., 2018). On the other hand, increases in GFAP expression have been observed in mice susceptible to depressive-like behaviors (Bravo-Tobar et al., 2021).

Therefore, in this collaborative study, we aimed to analyze the neuronal and synaptic morphological characteristics presented by individuals susceptible to the negative effects of social stress on the reinforcing actions of drugs, in this case alcohol. For this purpose, the brains of animals subjected to SD and evaluated in the behavioral paradigm of oral ethanol self-administration were sent to the IBCN at the UBA. Once there, both synaptic and neuronal morphology of the animals were analyzed in the hope of observing differences between animals classified as resilient and susceptible.

Specifically, I will present the results obtained in the behavioral tests and in the immuhistochemistry performed in the prelimbic cortex (PrL), striatum and CA1 of

the hippocampus to detect MAP2, NF200, GFAP and S100 calcium-binding protein β (S-100β) by immunofluorescence technique.

2. Methodology

2.1. Animals

A total of 75 male OF1 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents (n= 20 adult mice) were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998b). All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (white light from 8:00 to 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia.

2.2. Drugs

For the drinking in the dark and oral SA procedures, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a 20% (v/v) ethanol solution.

2.3. Experimental design

In the experimental design, all the animals were delivered to our laboratory PND 21 and were housed in regular condition throughout the study. Initially, two experimental groups were randomly assigned, on the one hand, a control group and a defeated group (which remained undisturbed until PND 47). Subsequently, all mice were exposed to the SD procedure or exploration from PND 47 to 56. 24 h after the

last SD episode, the animals performed the social interaction test (SIT) to assess depressive-like behaviors and were characterized as resistant or susceptible based on their social withdrawal coefficient (SWR). From this point on, the SD groups were divided into susceptible and resilient groups, forming three experimental groups: CRTL; SD-R; SD-S. Two weeks after the last defeat, the animals started the dinking in the dark (DID) test for four days and, in the following week, the animals started the ethanol SA protocol for approximately 22 days. At the end of this test, all animals were perfused for subsequent analysis of the different markers in PrL, striatum and hippocampus using the immunofluorescence technique.

The experimental design is depicted in Figure 1.

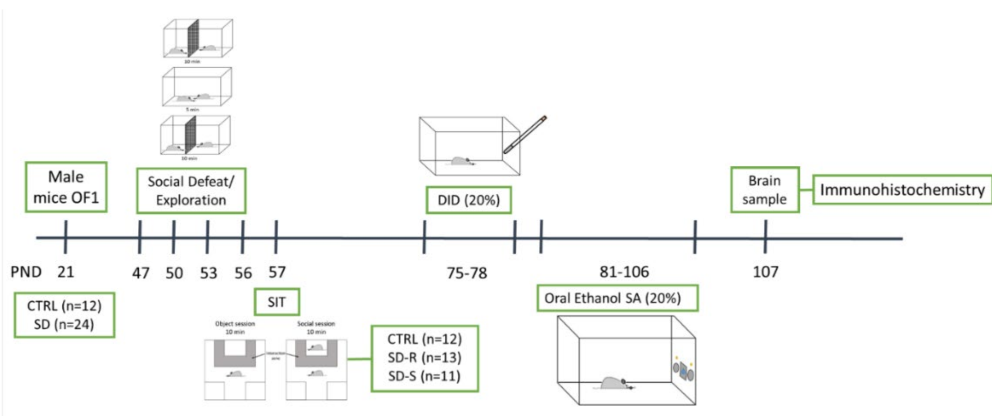

Figure 1. Experimental design

2.4. Procedure and apparatus

2.4.1. Procedure of social defeat (SD)

Animals in the stress/defeated groups were exposed to 4 episodes of SD during adulthood, each lasting 25 min and consisting of three phases. The initial phase began by introducing the “intruder” (the experimental animal) into the home cage of

the "resident" (the aggressive opponent) for 10 min (Tornatzky & Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-minute period. Finally, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al., 1998.

2.4.2. Social interaction test (SIT)

The SWR used was based on the social approach-avoidance test previously described by (Berton et al., 2006). The test took place 24 h after the last SD during daylight and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Animals were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10×6.5×35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the

cage as a social stimulus. Although it can be argued that the probe mouse used in the social interaction test (SIT) resembles the aggressor, and that this could foster social aversion, this is unlikely, since previous experiments demonstrate similar amounts of social investigation, irrespective of the strain used (Berton et al., 2006). Before each session, the arena was cleaned with 5 % alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Locomotion and arena occupancy during object and social sessions were determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage. Corners were defined as two squares of similar areas on the opposite wall of the arena.

2.4.3. Drinking in the dark (DID)

Following the basic paradigm of (Rhodes et al., 2005), the test consists of two phases. The first is habituation, where the animals are removed from their cages to be housed individually for one week to habituate them to the cages and the suction tubes containing a ball bearing at the end to prevent leakage, which will be used throughout the test. In the second phase of the protocol, the test begins 3 hours after lights out and the water bottles are replaced with 10 ml graduated cylinders containing a 20% (v/v) ethanol solution. These will remain in place for 2 hours. After this 2-hour period, the animals are returned to their grouped cages, with food and water bottles *ad libitum* again. This procedure is repeated on days 2 and 3, and on day 4, the procedure lasts for 4 h. In addition, immediately after each day, liquid

consumption is recorded. Fresh ethanol solution is prepared each day. In our case, we will maintain the protocol for two consecutive weeks, one for habituation and one for testing.

2.4.4. Oral ethanol self-administration (SA)

This procedure is based on that employed by (Navarrete et al., 2014). Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 20 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, fixed ratio 1 (FR1) and progressive ratio (PR) with a 20% ethanol concentration.

Training phase (12 days)

Mice were trained to respond to the active nose-poke to receive 20 µl of 20% (v/v) ethanol reinforcement. No food or water deprivation was performed in this protocol.

FR1 (10 days)

The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 20% ethanol (v/v) intake and the motivation to drink. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of effective

responses and ethanol consumption (µl) were measured under a fixed ratio 1 (FR1) for 10 daily consecutive sessions.

PR (1 day)

A PR session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40. All the sessions lasted one hour, except the PR session, which lasted two hours.

2.4.5. Tissue fixation and processing.

After 24 h after completion of oral ethanol SA, 27 mice (9 per group) were anesthetized with an intraperitoneal dose of 4% chloral hydrate. Each animal was perfused through the left ventricle of the heart initially with cold saline solution containing 0, 05% (w/v) NaNO2 (as a vasodilator) plus 1% (v/v) heparin 5000 IU/ml (as an anticoagulant) and subsequently with 300 ml of a cold fixative solution containing 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the skull and kept immersed in the same cold fixative solution for another 4 hours (postfixation period). The brains were then rinsed three times in 0.1 M phosphate buffer, pH 7.4, with 5% (w/v) sucrose, and left in this rinsing solution for 18 hours at 4°C. Brain sections (thickness: 40 µm) were cut with a cryostat (Leica, CM 1850); coronal sections of

the brain were cut. Brain sections were cryoprotected with 50% (w/v) sucrose in 0.1 M phosphate buffer, pH 7.4 and stored at -20°C, stored in 50% (v/v) glycerol solution at -20°C until use in immunohistochemical experiments.

2.4.6. Selection of tissue sections

Coronal serial sections with PrL, hippocampus and striatum were selected from 6 different mice from each group. Brain sections corresponding to a Bregma level of 2.46 to 1.70 mm for layer 5 of the PrL, a Bregma level of 0.98 to 0.02 mm for the striatum, and a Bregma level of -1.82 to -2.46 mm for the CA1 stratum radiatum (Franklin & Paxinos, 2008) were selected for the corresponding histological studies.

2.4.7. Immunohistochemistry

Two 40 µm-thick coronal brain sections containing the PrL, striatum, and hippocampus were randomly selected from six mice per group. The sections were washed three times in PBS and immersed in a solution of 3% (v/v) normal horse serum plus 0.5% (v/v) Triton X-100 in PBS for 3 h at 4°C under agitation to permeabilize and block nonspecific sites. The sections were then incubated with the following primary antibodies diluted in a solution of 1% (v/v) normal horse serum and 0 3% (v/v) Triton X-100 in PBS: mouse anti-MAP-2 (1:1.000, Sigma-Aldrich, Cat# M4403, RRID:AB_477193), mouse anti-NF200 (1:1,000, Sigma-Aldrich, Cat# N0142, RRID:AB_477257), mouse anti-S-100β (1: 1000, Sigma-Aldrich, Cat# S2532, RRID:AB_477499), rabbit anti-GFAP (1:3000, Agilent Cat# Z0334, RRID:AB_10013382).

The sections were incubated at 4°C overnight under agitation. After three washes in PBS, sections were incubated for 3 hours in the dark with fluorescent secondary antibodies: Alexa Fluor™ 568-conjugated anti-mouse IgG (1:1.000, Invitrogen, Cat# A11004, RRID:AB_143162) and Alexa Fluor™ 488-conjugated anti-rabbit IgG (1:1.000, Invitrogen, Cat# A11008, RRID:AB_143165). Sections were

subsequently counterstained with Hoechst 33342 (1:1.000, Sigma-Aldrich) to label nuclei, mounted on gelatin-coated slides and covered with 70% glycerol mounting medium.

2.4.8. Morphometric digital image analysis

Photographs were taken in an inverted Olympus IX83 microscope with an objective of 20×. Images were acquired using high-resolution digital monochromatic sCMOS Orca camera (Hamamatsu) and CellSens Dimension CS-DI-V1 software.

All measurements were performed on photomicrographs taken with the corresponding microscope and were analyzed by two blind operators. The PrL, striatum, CA1 striatum radiatum were the brain areas selected for morphometric studies, and all measurements were performed with ImageJ software (NIH1).

From immunostaining, the percentage of area covered by MAP-2- and NF200-positive fibers was measured, as well as the optical intensity of S-100β and GFAP expression in 20× primary magnification images.

2.5. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SWR. SWR is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups. To analyze acquisition of ethanol SA, a three-way ANOVA was performed with a between-subjects variables –SD with three

levels (CRTL, SD-S and SD-R)– and a within-subjects variable –Days, with ten levels of FR1–. The effects of SD on breaking point values and ethanol consumption during PR was analyzed by a one-way ANOVA, with a between-subjects variable –SD–.

Immunohistochemistry was analyzed using a one-way ANOVA with a between-subjects variable –SD, with three levels (CTRL, SD-S and SD-R).

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics v.26. Data were expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant.

3. Results:

3.1. Classification between susceptible and resilient mice according to their social withdrawal ratio

Following the social withdrawal ratio calculation criteria, the CTRL group (n=12) showed a mean ratio higher than 1. In the SD group of animals (n=24), 45.83% of the mice showed a social withdrawal ratio under 1, which classifies them as susceptible (SD-S) mice (n=11), and the remaining 54.17% of the mice showed a ratio equal to or higher than 1, which classifies them as resilient (SD-R) mice (n=13).

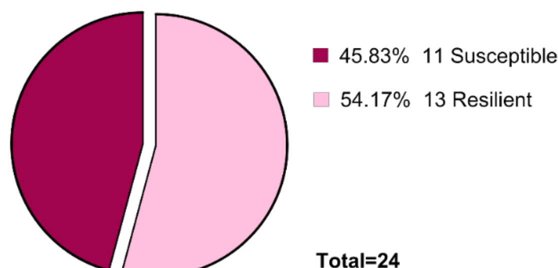

Figure 2. Percentages of resilient and susceptible animals among mice defeated during adulthood. The pie chart represents the percentage of resilient vs susceptible mice after social withdrawal ratio evaluation in the SIT.

3.2. Effect of social defeat on DID paradigm:

The ANOVA for ethanol consumption during DID revealed a significant effect of the variable Days [$F(3,99) = 38.639$; $p < 0.001$] (Fig.3). The post-hoc comparison showed that all mice consumed significantly more ethanol during day 4 of DID compared to days 1, 2 and 3 (p's < 0.001).

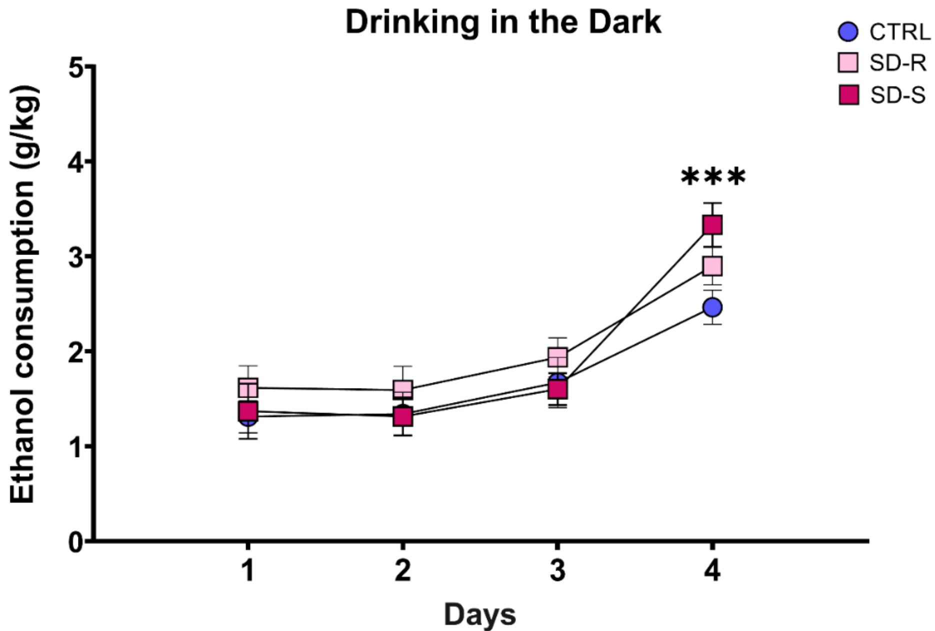

Figure 3. Effect of SD on ethanol intake during DID. Mice were divided into the following three groups: CTRL group allowed to explore a new cage (CTRL, n = 12); Resilient SD group exposed to social stress (SD-R, n=13); and Susceptible SD group exposed to social stress (SD-S, n=11). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of the g/kg of ethanol at 20% consumed. *** p<0.001 significant difference on day 4 compared to days 1, 2 and 3 in all mice.

3.3. Susceptible mice increased consumption and motivation for ethanol induced by social stress:

No differences were found between the animals during training phase, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the interactions Days × SD [$F(18,297) = 2.261$; $p = 0.003$] (Fig. 4a). The post-hoc comparison showed that CTRL group performed more active responses compared to SD-R group on day 2 ($p = 0.007$). Additionally, comparisons showed that in SD-S, mice performed fewer active responses on day 1 compared to day 5 ($p < 0.020$).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the interactions Days × SD [$F(18,297) = 1.941$; $p = 0.013$)] (Fig. 4b). The post-hoc comparison showed that the SD-S group consumed significantly more ethanol on days 5 ($p < 0.001$), 6, 7 (p's < 0.01), 8, 9 (p's < 0.05), 10 ($p < 0.01$) compared to the CTRL group, and on days 5 ($p < 0.01$), 6, 8 (p's < 0.05) and 10 ($p < 0.01$) compared to the SD-R group. Moreover, in the SD-S group, it was observed that a greater amount of ethanol was consumed on day 6 compared to days 1 ($p < 0.01$) and 2 ($p < 0.05$).

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the variable SD [$F(2,36) = 5.155$; $p = 0.011$] (Fig. 4c). The post-hoc comparisons showed that the SD-S group achieved a significantly higher BP than the CTRL and SD-R groups (p's < 0.05).

The ANOVA for ethanol consumption during PR revealed a significant effect of the variables SD [$F(2,36) = 3.656$; $p = 0.037$] (Fig.4d). The post-hoc comparisons

showed that SD-S group consumed significantly higher amounts of ethanol compared to CTRL group ($p < 0.05$).

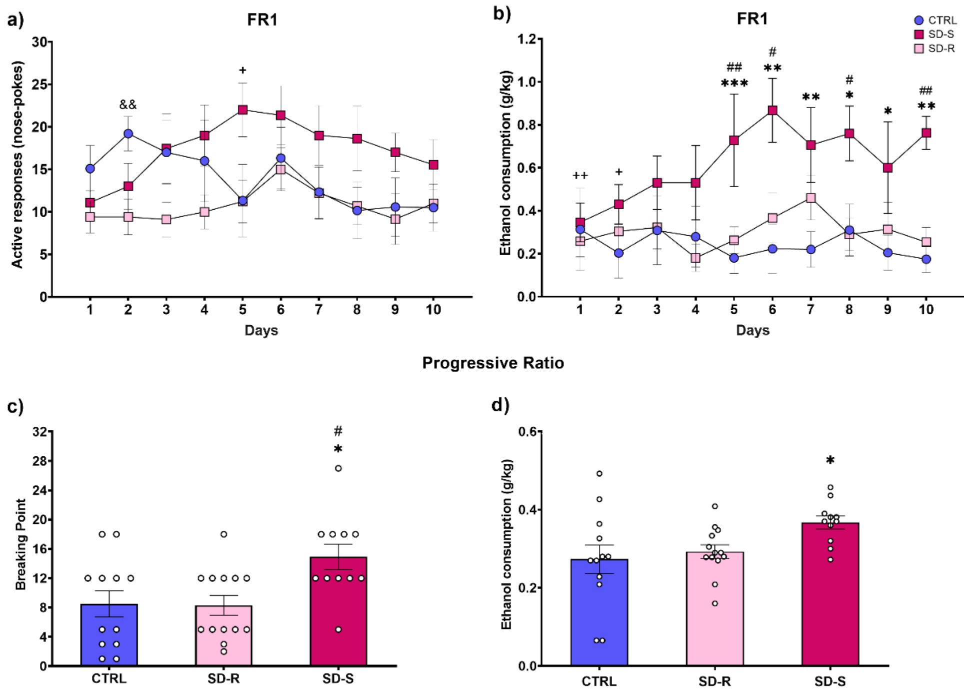

Figure 4. Effects of SD on the increase in oral ethanol self-administration OF1 mice. Mice were divided into the following three groups: CTRL group allowed to explore a new cage (CTRL, n = 12); Resilient SD group exposed to social stress (SD-R, n=13); and Susceptible SD group exposed to social stress (SD-S, n=11). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the g/kg of ethanol at 20% consumed during FR1 schedule. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, and (d) the g/kg of ethanol at 20% consumed during PR. * $p<0.05$, ** $p<0.01$ *** $p<0.001$ significant difference between susceptible mice vs. controls; # $p<0.05$, ## $p<0.01$ significant difference between susceptible mice vs. resilient mice; + $p< 0.05$, ++ $p< 0.01$ significant difference with day 6 in susceptible mice; && $p < 0.01$ significant difference between resilient mice vs. controls.

3.4. MAP-2 expression analysis

MAP-2 is a microtubule-associated protein whose expression is restricted to the neuronal cell body and dendrites (Dehmelt & Halpain, 2004). The MAP-2 protein participates in processes such as arborization and dendritic extension, being commonly used as a specific marker of dendrites.

The ANOVA for the PrL revealed a significant effect of the variable SD [$F(2,15) = 21.793$; $p < 0.001$] (Fig. 5d). The comparisons revealed a reduction in the area covered by MAP-2+ fibers in susceptible mice according with their SIT compared control and resilient mice (p's < 0.001).

The ANOVA for the striatum area revealed a significant effect of the variable SD [$F(2,15) = 14.369$; $p < 0.001$] (Fig. 5h). The comparisons revealed an increase in the area covered by MAP-2+ fibers in resilient mice according with their SIT compared control ($p < 0.05$) and susceptible mice ($p < 0.001$).

The ANOVA for the stratum radiatum of the hippocampal area CA1 revealed a significant effect of the variable SD [$F(2,15) = 8.794$; $p = 0.003$] (Fig. 5l). The comparisons revealed a reduction in the area covered by MAP-2+ fibers in susceptible mice according with their SIT compared control and resilient mice (p's < 0.01).

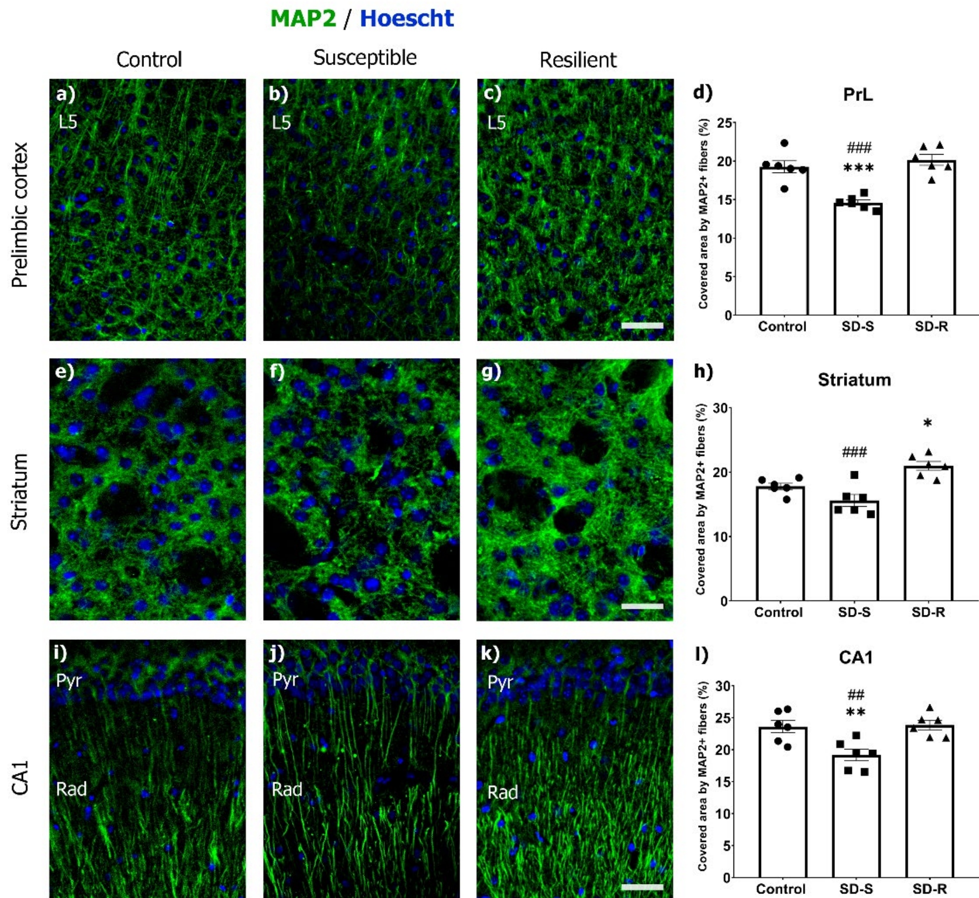

Figure 5. MAP2 expression. Immunofluorescence and semiquantification of area covered by MAP-2+ fibers in PrL (a, b, c, d), Striatum (e, f, g, h) and CA1 (i, j, k, l) from control (CTRL), resilient (SD-R) and susceptible (SD-S) mice (bars = 30 µm). L5: Layer 5; Pyr: pyramidal layer; Rad: stratum radiatum. The columns represent the mean and the vertical lines ± SEM (n = 6/group). ***$p < 0.001$, **$p < 0.01$, *$p < 0.5$ significant difference respect CTRL group. ###$p < 0.001$, ##$p < 0.01$ significant difference respect SD-R group.

3.5. Neurofilament expression analysis

Neurofilaments are the most important components of the axonal cytoskeleton and their elastic properties help maintain the markedly asymmetric shape of neurons. As

neurofilament dynamic remodeling is essential for axonal growth and maintenance, the study of neurofilament expression reveals whether alterations occur in neuronal functioning (Yuan et al., 2012).

The ANOVA for the PrL revealed a significant effect of the variable SD [$F(2,15) = 28.878$; $p < 0.001$] (Fig. 6d). The comparisons revealed a reduction in the area covered by NF200+ fibers in susceptible mice according with their SIT compared control and resilient mice (p's < 0.001). Moreover, resilient mice expressed a significant increase in area covered by NF200+ fibers in comparison of control mice ($p < 0.05$).

The ANOVA for the striatum area not revealed a significant effect of the variable SD [$F(2,15) = 0.598$; $p > 0.05$] (Fig. 6h).

The ANOVA for the stratum radiatum of the hippocampal area CA1 revealed a significant effect of the variable SD [$F(2,15) = 8.671$; $p = 0.003$] (Fig. 6l). The comparisons revealed a reduction in the area covered by NF200+ fibers in susceptible mice according with their SIT compared control and resilient mice (p's < 0.01).

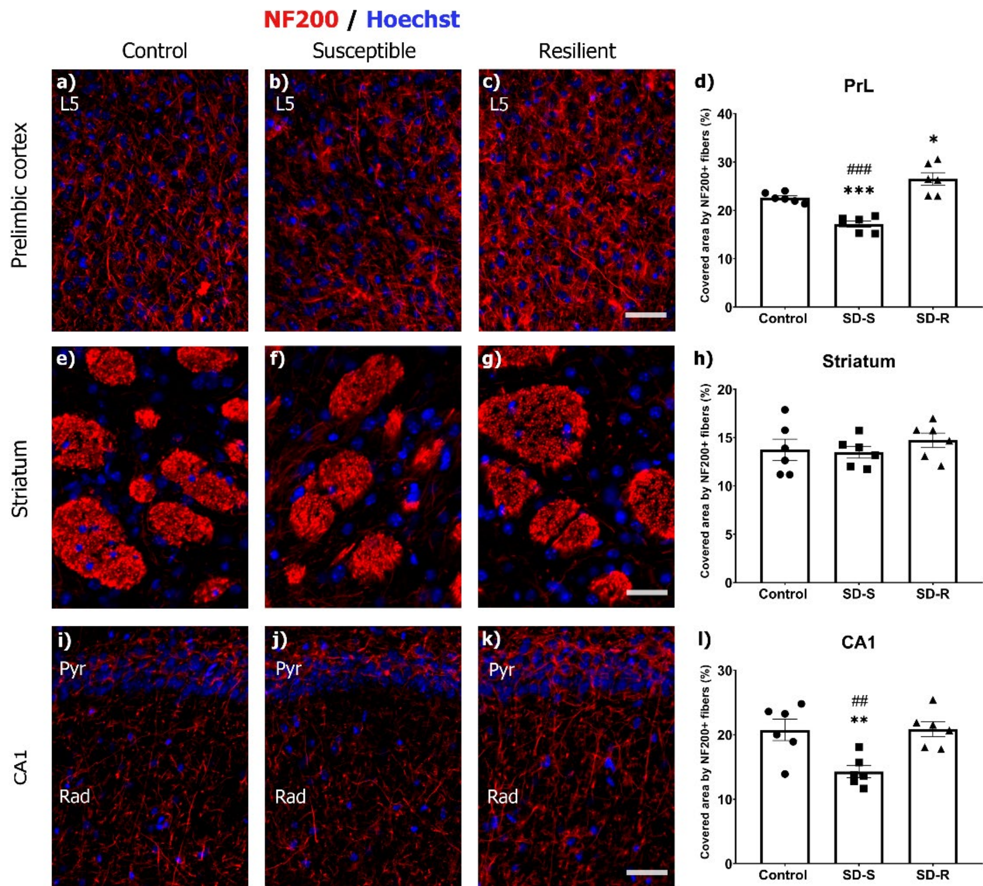

Figure 6. NF200 expression. Immunofluorescence and semiquantification of area covered by NF200+ fibers in PrL (a, b, c, d), Striatum (e, f, g, h) and CA1 (i, j, k, l) from control (CTRL), resilient (SD-R) and susceptible (SD-S) mice (bars = 30 μm). L5: Layer 5; Pyr: pyramidal layer; Rad: stratum radiatum. The columns represent the mean and the vertical lines ± SEM (n = 6/group). $^{**}p < 0.01$, $^{*}p < 0.5$ significant difference respect CTRL group. $^{###}p < 0.001$, $^{##}p < 0.01$ significant difference respect SD-R group.

3.6. Glial fibrillary acidic protein expression analysis

GFAP is one of the fibrous proteins that form the intermediate filaments of the intracellular cytoskeleton, particularly of glial cells such as astrocytes and Schwann cells (Middeldorp & Hol, 2011).

The ANOVA for the PrL revealed a significant effect of the variable SD [$F(2,15) = 5.196$; $p < 0.05$] (Fig. 7d). The comparisons revealed an increase in the area covered by GFAP+ astrocytes in susceptible mice according with their SIT compared resilient mice ($p < 0.05$).

The ANOVA for the striatum area revealed a significant effect of the variable SD [$F(2,15) = 17.476$; $p < 0.001$] (Fig. 7h). The comparisons revealed an increase in the area covered by GFAP+ astrocytes in susceptible mice according with their SIT compared to control and resilient mice (p's < 0.001).

The ANOVA for the stratum radiatum of the hippocampal area CA1 revealed a significant effect of the variable SD [$F(2,15) = 4.673$; $p = 0.026$] (Fig. 7l). The comparisons revealed an increase in the area covered by GFAP+ fibers in susceptible mice according with their SIT compared to resilient mice ($p < 0.05$).

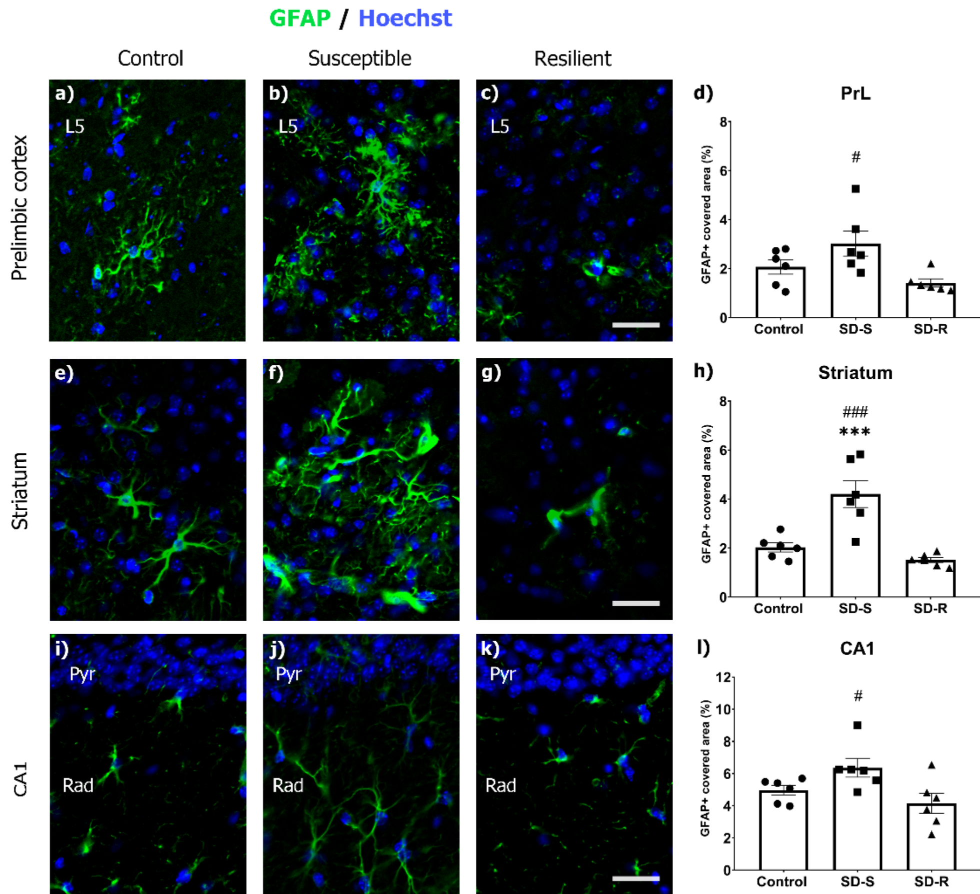

Figure 7. GFAP expression. Immunofluorescence and semiquantification of GFAP+ astrocytes area covered in PrL (a, b, c, d), Striatum (e, f, g, h) and CA1 (i, j, k, l) from control (CTRL), resilient (SD-R) and susceptible (SD-S) mice (bars = 30 µm). L5: Layer 5; Pyr: pyramidal layer; Rad: stratum radiatum. The columns represent the mean and the vertical lines ± SEM (n = 6/group). ***$p < 0.001$ significant difference respect CTRL group. ###$p < 0.001$, #$p < 0.05$ significant difference respect SD-R group.

3.7. S-100β expression analysis

This protein shows neurotrophic and neurite growth-promoting activities and is strongly involved in the development of several neurotransmitter systems (Donato et al., 2013). The ANOVA's for the PrL [$F(2,15) = 0.586$; $p > 0.05$] (Fig. 8d),

striatum area [F(2,15) = 0.377; p > 0.05] (Fig. 8h) and stratum radiatum of the hippocampal area CA1 [F(2,15) = 1.300; p > 0.05] (Fig. 8l) not revealed a significant effect of the variable SD.

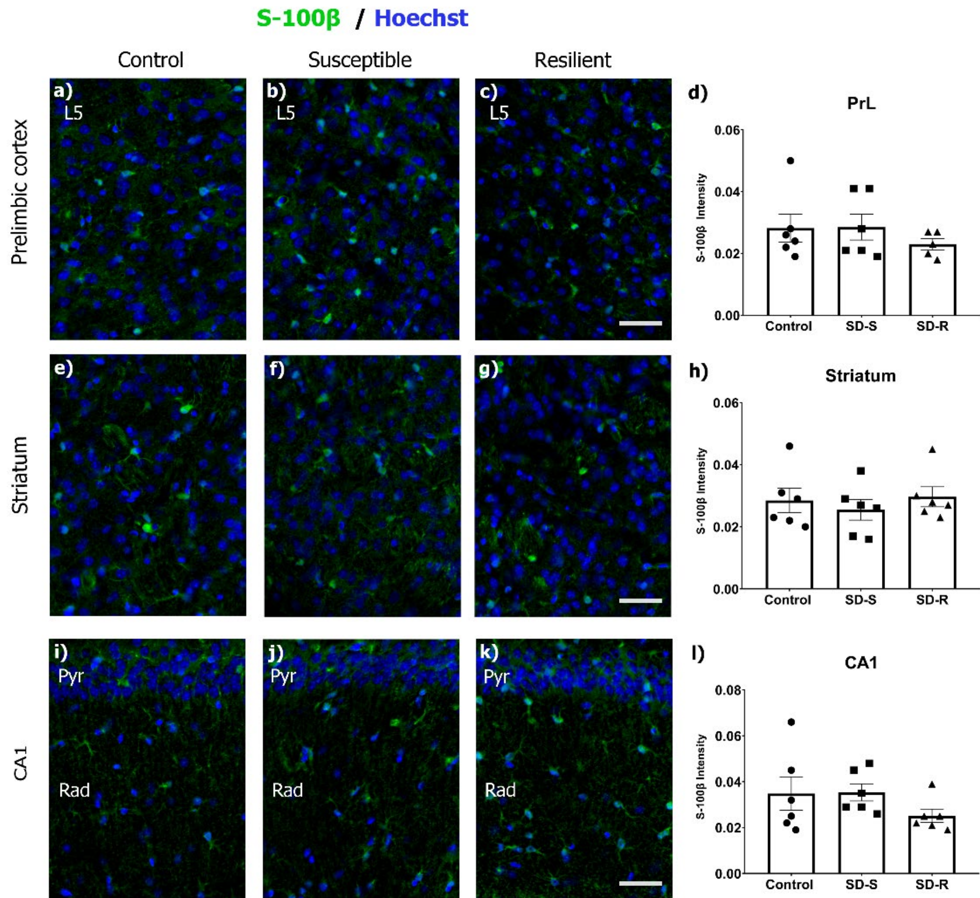

Figure 8. S-100β expression. Immunofluorescence and optical intensity of S-100β in PrL (a, b, c, d), Striatum (e, f, g, h) and CA1 (i, j, k, l) from control (CTRL), resilient (SD-R) and susceptible (SD-S) mice (bars = 30 µm). L5: Layer 5; Pyr: pyramidal layer; Rad: stratum radiatum. The columns represent the mean and the vertical lines ± SEM (n = 6/group).

4. Discussion

The present study corroborates the previously obtained results that SD produces depressive-like symptomatology in a specific population of defeated mice. The social interaction test was used to determine which mice are susceptible or resilient to depressive-like behaviors. In this study we obtained a percentage of the susceptible phenotype of 45%, which is similar to that obtained previously in our research group (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021a, 2022). These animals susceptible to SD showed a significant increase in ethanol consumption in the oral ethanol SA paradigm. In relation to synaptic and neuronal morphology, we observed morphological alterations in dendritic prolongations and axonal cytoskeleton. In addition, certain alterations in the neuronal gliosis response were also observed.

Respect to ethanol consumption, there is extensive literature demonstrating that exposure to social stress, such as the repeated SD used in this study, produces short- and long-term changes in ethanol intake in various paradigms (Hwa et al., 2016; Norman et al., 2015; Reguilón et al., 2020, 2021a, 2021b, 2022). Specifically, in SA, increases and escalations in consumption and increases in motivation to obtain the substance were observed following exposure to SD in susceptible mice. Also, increases in consumption induced by SD have been observed in other paradigms of voluntary ethanol consumption.

In relation to the markers targeted in this study, the scientific literature is not as extensive. In the present study, we observed in mice susceptible to depressive-like behaviors a decrease in the area covered by MAP-2+ fibers in PrL and CA1 of the hippocampus, as well as an increase in the area covered in the striatum of stress-resilient mice. The MAP-2 expression has been studied in some models of social stress. In humans, low levels of MAP-2 have been observed in hippocampal formations of patients with major depression (Soetanto et al., 2010). In preclinical

models of chronic SD stress, chronic unpredictable mild stress and maternal separation it was observed that defeated rodents suffered decreased MAP-2 gene expression in the hippocampus and cerebral cortex (Abdel-Rahman et al., 2004; García-Gutiérrez et al., 2016; Martin et al., 2017; Yang et al., 2015), this dendritic and synaptic alteration could lead to cognitive impairments (Abdel-Rahman et al., 2004; Martin et al., 2017), influence the development of depressive-like behaviors (García-Gutiérrez et al., 2016; Yang et al., 2015) and vulnerability to ethanol consumption during adolescence (García-Gutiérrez et al., 2016). Also, in the prenatal stress model in rats, a decrease in MAP-2 immunostaining levels in cortex, striatum and hippocampus was observed, this decrease in dendritic arborization could lead to a reduction of neuronal processes and a decrease in the number of synapses (Barros et al., 2006). The MAP-2 expression has also been studied in relation to ethanol consumption. Perinatal ethanol exposure, in chronic mild ethanol exposure in adolescents and adults, causes a decrease in dendritic arborization in cortical, hippocampal and striatal neurons in rats (Evrard et al., 2006; Guadagnoli et al., 2016; Tagliaferro et al., 2006). Furthermore, in vivo neuroimaging studies showed that ethanol administration produced a decrease in MAP-2 in male and female mice, related to neuronal injury, being much more evident in females (Alfonso-Loeches et al., 2013).

In relation to neurofilaments, we have observed in mice susceptible to depressive-like behaviors a decrease in the area covered by NF200+ fibers in PrL and CA1 of the hippocampus, as well as an increase in the area covered in the PrL of stress-resilient mice. Although there are fewer studies of the effects of social stress on neurofilament morphology, a decrease in expression was observed in mice subjected to maternal separation (García-Gutiérrez et al., 2016). NF200 plays a major role in the stabilization and maturation of pre-existing connections, a dysregulation of its expression can derive in a deterioration of synaptic connections (García-Gutiérrez et

al., 2016). Other studies have shown that alcohol exposure during early life and adulthood causes decreases in NF200, leading to alterations of the axonal cytoskeleton in hippocampus, striatum and frontal cortex (Evrard et al., 2006; Tagliaferro et al., 2006).

GFAP and S-100β are two proteins found in astrocytes and are widely implicated in functional processes of CNS cells and in the functioning of the blood-brain barrier (BBB). GFAP is involved in social stress-induced astrogliosis although the results in the scientific literature are mixed. In the present study, we observed only in mice susceptible to depressive-like behaviors a decrease in the area covered by GFAP+ fibers in PrL, striatum and CA1 of the hippocampus. In line with the results obtained in this study, in a study using SD in rats, an increase in GFAP fluorescence in the hippocampus, one and two weeks after SD was observed in animals with a phenotype susceptible to the effects of social stress (Bravo-Tobar et al., 2021). In contrast, a study assessing GFAP 48 hours after the last episode of chronic SD in male mice and chronic vicarious SD in females revealed no significant alterations of astrocyte activation in the VTA (Bali et al., 2021). Instead, three weeks of chronic SD showed reduced GFAP expression in the PFC and NAc in male and female rats, indicating that prolonged chronic social stress over time can induce astrocyte impairment (Rappeneau et al., 2016). Similar results were observed in the hippocampus of adult male rats subjected to 5 weeks of chronic SD (Araya-Callís et al., 2012). In relation to alcohol consumption, this is a widely researched topic. For example, in adult rodents, it is known that prolonged exposure to ethanol produces an increase in astrocyte reactivity with elevated GFAP expression in the hippocampus and cerebral cortex, promoting astroglial reaction to alcohol-induced brain injury (Alfonso-Loeches et al., 2013, 2014; Kane et al., 2014; Tagliaferro et al., 2006). Unfortunately, there are no studies evaluating GFAP expression in rodents subjected to SD and subsequently exposed to ethanol consumption. However, in a similar

study, in male mice subjected to repeated SD and subsequently exposed to cocaine conditioned rewarding effects, a long-lasting decrease of GFAP+ cells in the NAc and PFC has been observed (Rodríguez-Arias et al., 2018). Thus, the intensity of SD interferes with GFAP expression, hypothesizing that a strong social stress prolonged in time leads to astrocyte deterioration and therefore a malfunction of astrogliosis, and an intermittent or less intense social stress coupled with ethanol intake may lead to overexpression in the face of possible neuronal damage, neuroinflammation or BBB repair.

The final marker presented in this study is the S-100β protein, which is responsible for the regulation of cell proliferation and differentiation, apoptosis, calcium homeostasis, energy metabolism, inflammatory response, cell migration/invasion, and regulation of transcription factors. Although the relationship is complex, many clinical studies have correlated increases in serum S-100β levels with the severity of major depressive disorder and other mood disorders (Tural et al., 2021). In animal models, following exposure to chronic unpredictable stress in rats, a significant increase in S-100β levels was observed in hippocampus (Stroth & Svenningsson, 2015; Wang et al., 2016; Ye et al., 2011), serum and PFC (Luo et al., 2010). However, decreases of S-100β have also been observed in the hippocampus in rats subjected to chronic mild stress (Rong et al., 2010). The mechanism by which S-100β promotes depressive symptoms is unknown, but as already discussed there is a strong positive correlation between S-100β levels and depression, as well as, there appears to be a positive correlation of S-100β levels with the severity of depressive-like symptoms (Tural et al., 2021). Chronic exposure to ethanol at low concentrations during adolescence caused a decrease in S-100β in hippocampus, striatum and frontal cortex, which could be due to ethanol toxicity (Evrard et al., 2006) and may contribute to increases in GFAP expression (Chang et al., 2005; Evrard et al., 2006). However, the same protocol of ethanol administration revealed

an increase of S-100β immunostaining in the hippocampus in male adult rats (Tagliaferro et al., 2006), which the authors attribute to a decrease in S-100β release. In a study in adult rats chronically exposed to ethanol, only S-100β alterations were observed in the dentate gyrus of the hippocampus, showing an increase in this protein (Huf et al., 2019). On the other hand, increases in gene expression of this protein have been observed in the NAc of ethanol-preferring rats after exposure to operant self-administration of ethanol (Rodd et al., 2008). In our study, we have not observed alterations of S-100β fluorescence in any of the structures studied.

In conclusion, these results demonstrate that SD can produce changes in neuronal cytoarchitecture, which may affect the formation and stability of synaptic connections in mice with a susceptible phenotype. In contrast, the observed increase in the area covered by MAP-2+ fibers in the striatum and by NF200+ fibers in the PrL of resilient mice may indicate increased neuronal plasticity in these individuals. Furthermore, the astrogliosis response observed in this study indicates that repeated social stress and ethanol exposure may produce neuronal damage or increased neuroinflammatory response in individuals susceptible to social stress.

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5. CONCLUSIONES FINALES

5. Conclusiones finales

Los resultados obtenidos en la presente Tesis Doctoral nos permiten confirmar que la derrota social contribuye al desarrollo de conductas de evitación social e incrementa el consumo de etanol a largo plazo mediante la activación del sistema inmune. Hemos confirmado la existencia de fenotipos diferenciados en base a las respuestas conductuales de evitación social inducidas por la derrota social. El estrés social produce efectos negativos, pero no en todos los animales expuestos, ya que solo un porcentaje de ellos desarrolla un perfil susceptible a las conductas de tipo depresivo e incrementa el consumo de alcohol. En esta Tesis Doctoral hemos caracterizado a los animales susceptible y resilientes a los efectos de la derrota social, tanto cuando esta se produce en la edad adulta como cuando se experimenta en la adolescencia. Pero nuestro principal objetivo ha sido potenciar la respuesta de resiliencia y hemos demostrado que intervenciones farmacológicas como la administración de oxitocina o ambientales como el ejercicio físico pueden potenciar dicha respuesta resiliente. Pero estas intervenciones también pueden aplicarse con carácter preventivo durante la adolescencia. Así por ejemplo el ejercicio físico, el enriquecimiento ambiental o la exposición a un estresor social de baja intensidad antes de experimentar la derrota social inducen una potente respuesta resiliente. Todos estos efectos protectores pueden producirse por diversos mecanismos, algunos de ellos estudiados en esta Tesis Doctoral, como los cambios en el BDNF o en la arquitectura neuronal. Sin embargo, el mecanismo fundamental que aparece como común en los animales resilientes es una disminución de la respuesta neuroinflamatoria que se observa siempre incrementada en animales derrotados susceptibles.

A continuación, desarrollaremos las principales conclusiones derivadas de la presente Tesis Doctoral.

La derrota social incrementa el consumo y la motivación por el alcohol

Nuestros resultados indican que la derrota social incrementa la vulnerabilidad al desarrollo de conductas adictivas. Al igual que habíamos observado con la cocaína, los ratones derrotados muestran un incremento en el consumo de alcohol y un aumento de la motivación por conseguir una dosis de esta sustancia en el paradigma operante de autoadministración oral de etanol, en comparación con los ratones que fueron expuestos a la condición de exploración y que no experimentaros estrés social. Tanto durante las fases de ratio fijo 1 como 3, los animales derrotados consumieron más alcohol que los controles y realizaron un mayor número de respuestas efectivas. Igualmente, en la prueba de ratio progresiva realizaron un mayor trabajo para conseguir el alcohol que los animales controles. Todos estos efectos los hemos observado 3 semanas después de la última derrota social, por lo tanto, son efectos a largo plazo lo cual incrementa mucho más su valor traslacional puesto que nos indica que el estrés social es capaz de inducir modificaciones que perduran en el cerebro de estos animales a lo largo del tiempo y que van a incrementar la vulnerabilidad a desarrollar una conducta adictiva.

Finalmente, también hemos confirmado que estos efectos se observan igualmente en animales derrotados socialmente durante adolescencia y que se evalúan ya en la edad adulta. Por lo tanto, nuestro modelo ha demostrado ser igualmente válido en animales tanto adultos como adolescentes, lo cual significa que puede considerarse como un buen modelo de *bullying* o acoso escolar.

Los animales resilientes a las conductas depresivas inducidas por la derrota social también se muestran resilientes al incremento en el consumo de etanol cuando el estrés social ocurre en la edad adulta

Uno de nuestros principales resultados es la confirmación de la existencia de dos fenotipos diferenciados en base a la respuesta de evitación social inducida por la

derrota social. El estrés social produce efectos negativos, pero no en todos los animales expuestos, ya que solo un porcentaje de ellos desarrolla retraimiento social (evaluado por el SIT o test de interacción social) que se considera una conducta de tipo depresivo. Nuestros resultados han confirmado que aquellos animales susceptibles que desarrollan retraimiento social (depresión) también presentan un incremento en el consumo de etanol. Según los resultados obtenidos en los estudios que componen esta Tesis Doctoral, los ratones susceptibles al estrés social se caracterizaron por desarrollar una respuesta de evitación social mayor tras la derrota social, un aumento en la ingesta de alcohol y una mayor motivación por obtener esta sustancia. Mientras que los ratones resilientes a las conductas de tipo depresivos mostraron una mayor interacción social, menores niveles de consumo y de motivación por el alcohol.

Igualmente, los resultados obtenidos en esta Tesis Doctoral confirman y extienden resultados previos obtenidos en nuestro laboratorio describiendo el diferente afrontamiento que presentan los ratones resilientes y susceptibles durante el encuentro agonístico (Ballestín et al., 2021). Durante el encuentro agonístico, los ratones susceptibles sometidos a cuatro sesiones de derrota social adoptan un perfil de afrontamiento pasivo-reactivo durante los encuentros de derrota social, empleando más tiempo en comportamientos de huida/evitación y sumisión/defensa durante la derrota social. Los ratones derrotados susceptibles mostraron una disminución del tiempo de evitación y huida, y un incremento de las conductas de sumisión y defensa en el cuarto encuentro, lo que indica una falta de flexibilidad conductual ante la experiencia de derrota social ineludible (Hawley et al., 2010; Lambert et al., 2014). Por el contrario, en algunos grupos de animales hemos observado que los ratones clasificados como resilientes mostraron un menor afrontamiento pasivo-reactivo en comparación con el grupo susceptible, es decir, mostraron mayor afrontamiento activo (Ballestín et al., 2021). Aunque, se requieren

más estudios para concluir si las conductas realizadas durante los encuentros agonísticos pueden definir un perfil resiliente o los comportamientos pasivo-reactivos son generales en ambos fenotipos.

Como aportación altamente original, en esta Tesis Doctoral hemos observado que los ratones susceptibles también presentan alteraciones morfológicas en las prolongaciones dendríticas y en el citoesqueleto axonal, que podrían indicar un deterioro de conexiones sinápticas preexistentes y, a su vez, influir en el desarrollo de conductas de tipo depresivo y en la vulnerabilidad al consumo de alcohol. Por el contrario, en los ratones resilientes al estrés se observó un incremento del área cubierta de proteínas presentes en las prolongaciones dendríticas (MAP-2+) y en el citoesqueleto axonal (NF200+) en estriado y corteza prelímbica, respectivamente. Este hallazgo podría indicar una mayor plasticidad neuronal en los individuos resilientes. También, observamos ciertas alteraciones en la respuesta de la gliosis neuronal. La respuesta de astrogliosis observada nos indica que el estrés social repetido y la exposición al alcohol pueden producir daños neuronales o una mayor respuesta neuroinflamatoria en los individuos susceptibles al estrés social.

Los animales resilientes que experimentan la derrota social durante la adolescencia muestran un fenotipo mucho más complejo que los derrotados en la edad adulta

Un interesante resultado de esta Tesis Doctoral ha sido que la experiencia de la derrota social durante la adolescencia presenta características específicas que la diferencia de la experimentada durante la edad adulta. De acuerdo con los pocos estudios realizados en esta área, observamos que los ratones adolescentes derrotados no desarrollaron un fenotipo general de resiliencia/susceptibilidad. No hay correlación entre la resiliencia a la evitación social y el aumento de la respuesta al alcohol. Aunque el porcentaje de ratones resilientes/susceptibles tras la derrota social

es comparable al observado en los ratones adultos (según su respuesta de retraimiento social con el SIT), en los ratones resilientes al desarrollo de la evitación social se observó un aumento de la conducta ansiógena o de la ingesta de alcohol. Solo un 20% del total de los ratones derrotados fue resiliente a los efectos depresivos y el aumento de la ingesta de alcohol inducidos por derrota social. Estos resultados indican que la edad de exposición a la derrota social afecta al desarrollo de la resiliencia y que existe un desarrollo variable de la resiliencia entre los comportamientos de tipo depresivo y la respuesta a la recompensa de la droga. En consonancia con nuestros resultados, Alves-dos-Santos et al. (2020) observaron que los ratones adolescentes derrotados resilientes a la anhedonia o a la evitación social eran los ratones más afectados en términos de resultados endocrinos/fisiológicos (aumento de peso corporal y respuesta a la corticosterona). Asimismo, Vassilev et al. (2021) observaron que, en la adolescencia, la derrota social produce un deterioro del control inhibitorio independientemente de la evitación social.

La resiliencia debe considerarse como un proceso activo, que afecta tanto a las estrategias pasivas como a las activas, para lograr la mayor adaptación al estrés (Russo et al., 2012). Las respuestas en cada sistema particular pueden desarrollarse de forma diferente tras la exposición al estrés (Smith, 2019). Nuestros resultados sugieren que la derrota social durante la adolescencia conduce a un fenotipo propenso a la adicción en algunos ratones, que se manifiestan como resiliente durante el SIT. Aunque es necesario profundizar más en este tema, se han observado cambios en la conectividad dopaminérgica de la corteza prefrontal (Vassilev et al., 2021) que podrían estar relacionadas con la mayor respuesta a los efectos reforzantes del alcohol observados en los ratones adolescentes resilientes al estrés social.

Nuestros resultados confirman que, al contrario de lo que se suponía en los adultos, las respuestas al estrés de la derrota social son más complejas y singulares en los

adolescentes, por lo que hay que tener precaución en la correcta interpretación y traducción de estos fenotipos.

La administración de oxitocina y el ejercicio físico durante la exposición a la derrota social incrementan la respuesta de resiliencia

Una vez confirmada la existencia de dos subpoblaciones entre los individuos sometidos a derrota social y conocida la respuesta a los efectos reforzantes del etanol de cada una de ellas, nos propusimos investigar intervenciones que podrían potenciar la resiliencia para llevar a cabo el objetivo principal de la presente Tesis Doctoral, desarrollar estrategias farmacológicas y ambientales para mejorar la resiliencia a los efectos negativos inducidos por la derrota social sobre el comportamiento y la respuesta neuroinflamatoria.

Para ello, en primer lugar, nos propusimos evaluar intervenciones que actuaran durante las sesiones de derrota social. Por un lado, evaluamos el efecto de la administración de oxitocina antes de cada encuentro agonístico y, por otro, el efecto del ejercicio físico voluntario y controlado durante las sesiones de derrota social pero que en este caso continuara durante el resto del procedimiento experimental. Como primer y crítica condición en estos estudios, comprobamos que las derrotas sociales se produjeron de forma similar en aquellos animales tratados con oxitocina, así como en los expuestos al protocolo de ejercicio físico. Es decir, los animales residentes atacaron y amenazaron con la misma intensidad a los animales intrusos. Igualmente, ninguna de las intervenciones afectó a los comportamientos de evitación/huida y defensa/sumisión d ellos ratones intrusos. Pero lo realmente importante es que sí observamos diferencias posteriormente en el paradigma de la autoadministración oral de etanol. Los ratones derrotados socialmente, expuestos a estas intervenciones (ya sea la administración de oxitocina antes de cada derrota social o al ejercicio físico) mostraron un menor número de respuestas activas, un menor consumo de

alcohol y un menor esfuerzo y motivación por conseguir la droga en comparación con los roedores derrotados sin intervenciones.

El neuropéptido oxitocina actúa como neuromodulador del sistema nervioso central, interviene en una amplia variedad de interacciones sociales, tiene efectos ansiolíticos y atenúa la respuesta del eje hipotálamo-hipofisario-suprarrenal al estrés, promoviendo un aumento de la interacción social y disminuyendo la anhedonia y la evitación social que caracteriza al estrés social (Borland et al., 2018; Ebert & Brüne, 2018; Ferrer-Pérez et al., 2019a; Lukas et al., 2011; Nasanbuyan et al., 2018; Steinman et al., 2016; Wang et al., 2018; Winter & Jurek, 2019). Además, se ha observado que la administración de oxitocina disminuye el consumo de drogas, los síntomas de abstinencia y las conductas de búsqueda asociadas a diversas drogas de abuso (Leong et al., 2018; Pedersen, 2017). Por tanto, la administración de oxitocina previa a la exposición a la derrota social podría actuar evitando la sensibilización del sistema de recompensa y así reduciendo los efectos reforzantes del alcohol, por ejemplo, debido a su efecto atenuante sobre la respuesta al estrés.

El ejercicio físico moderado y controlado parece ser un modulador de las funciones mentales superiores. En roedores produce una mejora del aprendizaje, de la neurogénesis, de la angiogénesis, un aumento de los factores neurotróficos induciendo cambios en varias moléculas de señalización, así como reduciendo las conductas asociadas al estrés (Salam et al., 2009; Mul, 2018). Se ha observado que el ejercicio físico tras la derrota social reduce la evitación social y la anhedonia en roedores (Mul et al., 2018; Watanasriyakul et al., 2018; Zhang et al., 2019). Además, el ejercicio físico regula algunos componentes del eje hipotálamo-hipofisario-suprarrenal, generando una respuesta adaptativa al estrés (Pietrelli et al., 2018) y se han observado disminuciones en la ingesta de alcohol cuando se tenía acceso a las ruedas de ejercicio (Ehringer et al., 2009; Darlington et al., 2014, 2016). Se puede concluir que nuestros resultados confirman que el ejercicio físico voluntario y

controlado es una herramienta eficaz para prevenir y reducir los efectos perjudiciales relacionados con la conducta adictiva inducidos por el estrés social, fomentando una respuesta neuroendrocrina adaptativa y promoviendo factores neutróficos.

La resiliencia puede potenciarse antes de que se produzca la derrota social

En una serie de estudios posteriores nos propusimos evaluar si determinadas intervenciones ambientales podrían ser útiles como herramientas preventivas y potenciar la respuesta resiliente al estrés. Para evaluar el carácter potenciador y preventivo de estas intervenciones ambientales, todas ellas se realizaron durante el periodo de la adolescencia y finalizaron antes del inicio de la primera exposición a la derrota social, en el día postnatal 47, momento en que se considera a los roedores como jóvenes adultos. Para realizar este objetivo, evaluamos tres condiciones ambientales que tradicionalmente han demostrado ser útiles para controlar los efectos nocivos del estrés: el enriquecimiento ambiental, el ejercicio físico voluntario y la inoculación de estrés.

El enriquecimiento ambiental y el ejercicio físico previo a las derrotas sociales durante la adolescencia no aumentó el porcentaje de ratones resilientes a las conductas de tipo depresivo evaluadas el SIT tras la derrota social. Por el contrario, sí observamos un ligero aumento del porcentaje de ratones resilientes en el grupo de inoculación de estrés. En relación con el consumo de alcohol, las tres intervenciones fueron eficaces para reducir el consumo y la motivación por el alcohol en los animales adultos. En la prueba del *Drinking in the Dark* observamos una disminución del consumo de alcohol de forma generalizada en los grupos de animales expuestos a ejercicio físico y en los grupos expuestos a inoculación de estrés durante la adolescencia. En el paradigma operante de autoadministración oral de etanol, se observó que las tres intervenciones eran eficaces para reducir las respuestas activas, el consumo voluntario y el esfuerzo y la motivación para

conseguir una dosis de alcohol tanto en los ratones resilientes como susceptibles expuestos a las diferentes intervenciones.

El enriquecimiento ambiental se ha asociado típicamente con una mejora del bienestar, un aumento de la función cognitiva y una potenciación de la resiliencia al estrés. Se han utilizado diferentes modelos para reducir la vulnerabilidad a los efectos perjudiciales de la derrota social, pero los resultados observados en la literatura científica son contradictorios. Algunos estudios han observado aumentos en la agresividad y la ansiedad, con incrementos en los niveles de factor liberador de corticotropina (McQuaid et al., 2013a, 2013b). Sin embargo, otros estudios han mostrado una reducción de la ansiedad, de las alteraciones cognitivas y de los niveles de corticosterona en animales alojados en situación de enriquecimiento ambiental (Bahi, 2017; Branchi et al., 2013; Cordner & Tamashiro, 2016; Dandi et al., 2018; Marianno et al., 2017; Mesa-Gresa et al., 2016; Reichmann et al., 2013). En nuestro laboratorio hemos observado que el tipo de casas y tubos utilizados en el enriquecimiento puede determinar un incremento del estrés en los animales. Debemos tener en cuenta que la edad de los roedores influye en la estabilidad social, ya que a mayor edad y maduración sexual se presentan conductas más agresivas y dominantes en los ratones macho. En cambio, durante el periodo de adolescencia, los ratones, al igual que los humanos, buscan una mayor interacción social y la jerarquía social propia de la adultez aún no se ha consolidado (Bell, 2018; Lo Iacono & Carola, 2018). Por este motivo, consideramos apropiada la utilización de un modelo de enriquecimiento ambiental durante la adolescencia, con el objetivo de moldear un cerebro aún en desarrollo y promover respuestas más resilientes. El enriquecimiento ambiental promueve el desarrollo de respuestas neuroendocrinas adaptativas al estrés social, lo que indica la importancia de la exposición a entornos complejos durante la adolescencia.

Como se ha discutido en el apartado anterior, el ejercicio físico es capaz de modular factores neurotróficos que inducen cambios positivos y reduce la activación del eje hipotálamo-hipofisario-suprarrenal. Teniendo en cuenta estos datos, podemos hipotetizar que estas neuroadaptaciones inducidas en el eje hipotálamo-hipofisario-suprarrenal y en los factores neurotróficos se mantienen en el tiempo y median en la repuesta adaptativa al estrés y en el buen funcionamiento del sistema de la recompensa. Gracias a los resultados de esta Tesis Doctoral, podemos confirmar que la pre-exposición voluntaria al ejercicio físico es eficaz para evitar el aumento del consumo y la motivación por el alcohol inducido por la derrota social a largo plazo.

Por otro lado, y a pesar de sus consecuencias negativas, el estrés puede presentar un efecto adaptativo, ya que promueve la homeostasis (Selye, 1975a, 1975b). La exposición a eventos estresantes que no son devastadores, pero que son lo suficientemente desafiantes como para provocar la instigación emocional y el procesamiento cognitivo, puede promover el afrontamiento exitoso de los estresores posteriores (Levine, 1957; Levine et al., 1967; Lyons et al., 2010; Meichenbaum, 2017). Los resultados obtenidos en relación con la exposición a inoculación de estrés durante la adolescencia corroboran la hipótesis de que la inoculación de estrés puede ser un factor protector ante futuras exposiciones a un estresor de mayor intensidad. Basándonos en la poca bibliografía referente a este modelo, hipotetizamos que la inoculación de estrés durante la adolescencia podría aumentar la adaptación al estrés social en cuanto al desarrollo de comportamientos de tipo depresivo en la edad adulta. La inoculación de estrés actuaría como un factor protector, como un mecanismo que prepara al individuo y ayuda en la regulación homeostática del organismo ante futuras exposiciones a estresores de mayor intensidad. Nuestros resultados han confirmado que este efecto protector se manifiesta sobre todo disminuyendo el consumo de alcohol en la edad adulta.

La neuroinflamación parece mediar la respuesta de resiliencia al estrés social

De manera consistente, en todos los estudios que componen esta Tesis Doctoral, hemos confirmado que la derrota social puede contribuir a potenciar las propiedades gratificantes y motivacionales del alcohol a largo plazo mediante la activación temprana del sistema inmune, provocando un estado de neuroinflamación que contribuye al desarrollo de un perfil vulnerable al trastorno por abuso de sustancias (Tabla 1).

Hemos observado como la derrota social repetida induce un incremento de la respuesta neuroinflamatoria. La derrota social incrementa los niveles de las quimiocinas CX3CL1 y CXCL12 en el estriado de los ratones OF1 tras la cuarta derrota social. Además, este efecto sobre la respuesta neuroinflamatoria se mantuvo a largo plazo, ya que los ratones sometidos a derrota social presentaron incrementos de los niveles de ambas quimiocinas en el estriado tras finalizar el paradigma de la autoadministración oral de etanol.

Como ya se ha mencionado, no todos los sujetos experimentales presentaron un fenotipo susceptible al estrés social. Un porcentaje de los ratones es resiliente a las respuestas conductuales, pero, también al incremento en la neuroinflamación inducida por la derrota social. Observamos de forma generalizada incrementos significativos de los niveles de la citocina proinflamatoria IL-6 en estriado y corteza prefrontal solo en los ratones clasificados como susceptibles a las conductas de tipo depresivas inducidas por la derrota social. Por otro lado, en estos estudios hemos observado ciertas discrepancias en la cepa C57BL/6 en relación con la concentración de los niveles de la quimiocina CX3CL1. No existe un consenso claro sobre si la CX3CL1 es una quimiocina anti-inflamatoria o proinflamatoria. La CX3CL1 señaliza a través de su receptor Cx3cr1 que solo se expresa en la microglía, siendo crítico para la comunicación cruzada microglía-neurona. Los cambios inducidos por

el estrés social en CX3CL1 no están claros, algunos estudios reportan disminuciones de la expresión de CX3CL1 en corteza prefrontal, en estriado, hipocampo e hipotálamo (Ballestín et al., 2021; Montagud-Romero et al., 2020; Rosseti et al., 2016; Wholeb et al., 2013) e incrementos de la expresión en el hipocampo dorsal, corteza orbitofrontral y núcleo paraventricular del hipotálamo (Bollinger et al., 2017; Rossetti et al., 2016; Wu et al., 2020). En primer lugar, hemos observado diferencias entre la respuesta a esta quimiocina tras la derrota social dependiendo de la cepa de ratón utilizada. Se han observado incrementos en ratones de la cepa OF1, pero disminuciones en aquellos pertenecientes a la cepa C57BL/6. Para comparar estos estudios, debemos tener en cuenta las diferencias entre estas cepas con respecto a su respuesta al estrés. Los machos OF1 son mucho más territoriales, siendo su repercusión fisiológica mayor tras la derrota. Pero, también observamos además discrepancia en cuanto a la respuesta a los niveles de CX3CL1 en animales C57BL/6 tras la derrota social. Mientras que en el estudio 3 observamos una disminución de los niveles de CX3CL1 en estriado y corteza prefrontal en los ratones susceptibles, por el contrario, en el estudio 5 no observamos diferencias en el estriado, pero sí un incremento de la concentración de CX3CL1 en la corteza prefrontal de los ratones susceptibles. Estos resultados contradictorios confirman la necesidad de continuar estudiando el papel y el funcionamiento de esta interesante quimiocina.

En relación con la respuesta neuroinflamatoria observada en los ratones expuestos a derrota social repetida durante la adolescencia, debemos destacar que, igualmente se observaron aumentos en la concentración de IL-6 y CX3CL1 principalmente en el estriado de los ratones susceptibles. Estos resultados junto a los resultados obtenidos en el estudio 5 en relación con la respuesta neuroinflamatoria del grupo control sometido a inoculación de estrés durante la adolescencia, nos permiten concluir que el estrés social, además de sensibilizar el sistema mesolímbico, sensibiliza el sistema inmunológico durante la adolescencia. Por lo que la derrota social induce un estado

neuroinflamatorio a largo plazo independientemente de la edad de exposición al estrés social.

	Cepa	Tratamiendo	Fenotipo		Medida	Estructura	Momento temporal
Ratones adultos	OF1	Derrota social		↑	CX3CL1 CXCL12	Estriado	Tras 4ª derrota y autoadministración oral
		Oxitocina + Derrota		↓	CX3CL1		Tras 4ª derrota y autoadministración oral
					CXCL12		Tras 1ª, 4ª derrota y autoadministración oral
		Rueda + Derrota		↓	CX3CL1 CXCL12		Tras la autoadministración oral
	C57BL/6J	Derrota social	Susceptible	↑	IL-6	Estriado Corteza Prefrontal	Tras la autoadministración oral
				↓	CX3CL1		
			Resiliente	=	IL-6 CX3CL1		
		Enriquecimiento ambiental + Derrota	Susceptible	↓	IL-6	Estriado	
				↑	CX3CL1		
			Resiliente	=	IL-6 CX3CL1		
		Inoculación de estrés + Derrota	Susceptible	↓	IL-6	Estriado Corteza	
				=	CX3CL1	Estriado	
				↓		Corteza Prefrontal	
			Resiliente	↓	IL-6	Estriado Corteza Prefrontal	
				=	CX3CL1		
Ratones adolescentes	C57BL/6J	Derrota social	Susceptible	↑	IL-6	Estriado	Tras la autoadministración oral
				=		Corteza Prefrontal	
				↑	CX3CL1	Estriado	
				=		Corteza Prefrontal	
			Resiliente	=	IL-6 CX3CL1	Estriado y Corteza Prefrontal	

Tabla 1. Respuesta neuroinflamatoria. Comparación entre los cambios en la respuesta neuroinmune inducidos por la derrota social y las intervenciones con potencial para promover la respuesta resiliente. (↑) Incrementos; (↓) disminuciones; (=) sin cambios observados en comparación con el grupo control.

Entre las numerosas aportaciones de esta Tesis Doctoral, quizá consideramos la más relevante la confirmación de que el sistema inmune podría ser un mecanismo clave

mediante el cual los efectos de la derrota social inducen vulnerabilidad en el desarrollo de conductas adictivas. Pero, aún más importante es que mediante la aplicación de diferentes intervenciones hemos podido revertir y evitar la activación de esta respuesta neuroinflamatoria inducida por la derrota social. Numerosos estudios asocian a la **oxitocina** como un modulador de la respuesta neuroinflamatoria inducida por la derrota social. La oxitocina inhibe mediadores pro-inflamatorios como el TNF-α, la IL-1β, COX-2 y el óxido nítrico sintasa (Akman et al., 2015; Inoue et al., 2019; Karelina et al., 2011; Yuan et al., 2016). En concordancia con lo observado en la literatura científica, mediante la administración previa de oxitocina en cada derrota social, conseguimos bloquear el incremento de los niveles de las proteínas CX3CL1 y CXCL12 en el estriado inducido por la derrota social. Esta disminución de los niveles de concentración se mantuvo en el tiempo, tras el paradigma de la autoadministración oral de etanol. Por tanto, podemos confirmar que la administración exógena de oxitocina tiene efectos antiinflamatorios sobre la respuesta neuroinflamatoria desencadenada por la derrota social.

La literatura científica sugiere que el **ejercicio físico** de intensidad moderada puede ser óptimo para disminuir los marcadores neuroinflamatorios (Henrique et al., 2018; Paolucci et al., 2018). Algunos estudios han mostrado los efectos positivos del ejercicio físico controlado sobre el estrés (Mul et al., 2018; Ignácio et al., 2019) y la conducta adictiva (Somkuwar et al., 2016). El ejercicio físico interactúa con el estrés y la neuroinflamación en función de la intensidad. Varios estudios han observado que el ejercicio físico controlado reduce los niveles de corticosterona y los receptores de glucocorticoides, atenuando los efectos negativos del estrés crónico (Zheng et al., 2006; Ignácio et al., 2019; Lynch et al., 2019; Watanasriyakul et al., 2019). La inhibición del exceso de producción de corticosterona puede atenuar la respuesta inflamatoria inducida por el estrés (Niraula et al., 2018). En esta Tesis Doctoral, demostramos que la exposición a ejercicio físico voluntario y controlado durante todo el procedimiento experimental es efectivo para revertir el aumento de

los niveles de CX3CL1 y CXCL12 en el estriado inducido por derrota social repetida tras la autoadministración oral de etanol. Por lo tanto, el ejercicio físico voluntario y controlad es una herramienta eficaz para prevenir y revertir la activación del proceso neuroinflamatorio inducido por la derrota social.

Además, observamos un efecto preventivo en la activación de la respuesta neuroinflamatoria en los roedores susceptibles que fueron expuestos a **enriquecimiento ambiental**. Observamos un efecto generalizado de disminución en la concentración de IL-6 en el estriado en todos los ratones expuestos a enriquecimiento ambiental. Además, en los ratones susceptibles a los efectos de la derrota social expuestos a enriquecimiento ambiental se observó una disminución de los niveles de CX3CL1 en el estriado en comparación con los ratones susceptibles sin enriquecimiento ambiental. Aunque no existen estudios similares, estos resultados van en la línea de un estudio que evaluó el efecto del enriquecimiento ambiental en la respuesta neuroinflamatoria inducida por estrés social moderado, observándose una atenuación del aumento de la expresión del ARNm prefrontal de IL-6 e IL-β1 (McQuaid et al., 2018). Estos resultados sugieren que la exposición a enriquecimiento ambiental antes de la derrota social reduce el impacto del estrés social a largo plazo en la respuesta neuroinflamatoria, actuando como un factor protector.

Por otro lado, evaluando la **inoculación de estrés** durante la adolescencia, observamos una disminución generalizada de los niveles de IL-6 en la corteza prefrontal de todos los ratones expuestos a inoculación de estrés en comparación a todos los ratones no inoculados. Adicionalmente, en la corteza prefrontal se observó una disminución de esta citocina entre los ratones susceptibles inoculados con estrés en comparación con los ratones susceptibles no inoculados. Con respecto a la quimiocina CX3CL1, también observamos un papel protector de la inoculación de estrés, ya que observamos una disminución de los niveles en el grupo susceptible

expuesto a inoculación de estrés en comparación con el grupo susceptible no expuesto a inoculación en la corteza prefrontal. Además, se observaron diferencias significativas entre los grupos control, con un aumento de los niveles de CX3CL1 en el grupo control expuesto a inoculación de estrés en comparación con el grupo control sin inoculación de estrés. Por el contrario, no se observaron diferencias estadísticas en el estriado. Aunque no existen estudios que apoyen nuestros resultados, consideramos que el modelo de inoculación de estrés es realmente prometedor para prevenir la activación de la respuesta neuroinflamatoria inducida por estrés social, ya que la inoculación de estrés podría inducir una menor reactividad del eje HPA a una experiencia de estrés posterior y/o al desarrollo de una mayor neuroplasticidad o flexibilidad conductual que caracteriza al fenotipo resiliente (Hawley et al., 2010; Lambert et al., 2014; Lee et al., 2014; Macrí et al., 2011). Estos resultados indican que entrenando a los individuos ante la exposición a situaciones estresantes se pueden desarrollar respuestas adaptativas al estrés social.

Limitaciones y estudios futuros

Aunque en esta Tesis Doctoral hemos podido dar respuesta a algunas preguntas muy relevantes, no se encuentra exenta de limitaciones. El fenómeno de la resiliencia es un concepto que aún se encuentra en pleno proceso de investigación, los mecanismos neurobiológicos subyacentes a este fenómeno aún son desconocidos y es necesario realizar más investigaciones y superar algunas limitaciones presentadas en este trabajo. A continuación, se exponen las cuestiones abiertas y los nuevos retos que podrían abordarse en futuros estudios.

Actualmente, sabemos que existen diferencias de género/sexo en la respuesta al estrés y en la vulnerabilidad a trastornos relacionados con el estado de ánimo y a la adicción. Consideramos imprescindible para paliar la principal limitación de este trabajo, y así, ampliar el conocimiento sobre las bases neurobiológicas que subyacen

a la resiliencia al estrés y como siguiente paso en la ampliación de esta Tesis Doctoral, la realización de estos estudios en roedores hembra. Actualmente en nuestro laboratorio hemos desarrollado un protocolo de derrota social vicaria en hembras que ha producido efectos muy similares a los observados en machos.

En segundo lugar, proponemos seguir estudiando el potencial preventivo de intervenciones ambientales para potenciar la resiliencia en los roedores adolescentes. En este sentido, hemos demostrado que la caracterización del fenotipo resiliente durante la adolescencia es diferente al presentado en los ratones adultos. La respuesta al estrés por derrota social parece ser compleja y singular en los adolescentes, y se debe ser cuidados en la interpretación y traducción de los fenotipos resilientes/susceptibles. No podemos presuponer que la resiliencia a un fenotipo se desarrolla igualmente para otros cuando se trata de animales adolescentes. En futuros estudios pretendemos desarrollar estrategias para potenciar la resiliencia al estrés social experimentado durante la adolescencia. Algunos de los objetivos preliminares son el estudio de la inoculación de estrés en etapas previas al destete de los ratones mediante la deprivación maternal breve, o la aplicación de ambientes enriquecidos (jaulas más grandes con casitas, tubos de PVC, material extra de anidación, etc.,).

Valor traslacional de este estudio

Los modelos animales son una potente herramienta para realizar investigaciones en diversos ámbitos. En nuestro caso la utilización de modelos animales es fundamental para investigar las bases neurales y fisiológicas del estrés y la adicción a drogas. Sin embargo, cuando nos enfrentamos a unos resultados obtenidos gracias al uso de modelos animales debemos ser prudentes a la hora de trasladarlos a la conducta humana. La literatura coincide en señalar que el estrés puede ser desencadenante de diversas patologías en los humanos, como estados depresivos, ansiosos e incluso trastornos adictivos (Chen et al., 2012; Hymel et al., 2014; Sinha, 2008). Los

resultados obtenidos con el modelo de derrota social pueden extrapolarse a los humanos en situaciones de estrés psicológico o social a las que estamos expuestos durante gran parte de nuestra vida. Estas situaciones estresantes producen cambios a nivel del eje hipotálamo-hipofisario-adrenal al igual que ocurre en los animales y alteran la respuesta de los individuos ante las drogas. Nuestros resultados son muy interesantes si pensamos no solo en una diana terapéutica que pueda reducir los cambios neurofisiológicos que produce el estrés y evitar los cambios que se relacionan con el abuso de drogas como el alcohol, sino también, sino también como estrategias preventivas que fomenten el desarrollo de respuestas resilientes a los efectos adversos que induce el estrés social. Además, nuestros resultados proporcionan pruebas conductuales y neurobiológicas de que la exposición a la derrota social durante una etapa temprana de la vida, como la adolescencia, modifica la vulnerabilidad al abuso de sustancias, sensibilizando de forma temprana el sistema inmune. Considerando de vital importancia fomentar y ofrecer mecanismos que favorezcan respuestas de afrontamiento positivo y activo para evitar o reducir las consecuencias negativas que produce el estrés social.

Los resultados que se han presentado en esta Tesis Doctoral pueden ayudar a la identificación de los sujetos más vulnerables a las conductas de tipo depresivo o al consumo de drogas tras la exposición al estrés social. Pero aún más importante, hemos señalado una serie de intervenciones que van a potenciar la respuesta resiliente, ya sea aplicadas durante la adolescencia y previas a experiencias vitales estresantes o durante los episodios de estrés. De esta manera se contribuye al desarrollo de terapias individualizadas con carácter preventivo y terapéutico en el tratamiento de trastornos adictivos.

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ANNEXES

Annex 1: Study 1.
Oxytocin reverses ethanol consumption and neuroinflammation induced by social defeat in male mice

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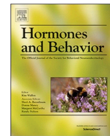

Oxytocin reverses ethanol consumption and neuroinflammation induced by social defeat in male mice

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A B S T R A C T

Oxytocin (OXT) modulates social interactions, attenuates stressful responses and can decrease drug-seeking and taking behaviors. In previous studies, we observed that social defeat (SD) induced a long-lasting increase in ethanol intake and neuroinflammation in male mice. We also know that OXT blocks the increase in cocaine reward induced by SD. Therefore, in the present study we aimed to evaluate the effect of 1 mg/kg of OXT administered 30 min before each episode of SD on ethanol consumption and the neuroinflammatory response in adult male mice. Three weeks after the last SD, mice underwent oral ethanol self-administration (SA) procedure, and striatal levels of the two chemokines CX3CL1 and CXCL12 were measured after the last SD and at the end of the ethanol SA. OXT administration blocked the increase in voluntary ethanol consumption observed in defeated mice, although it did not affect motivation for ethanol. An increase in the striatal levels of CX3CL1 and CXCL12 was observed in defeated animals immediately after the last defeat and after the ethanol SA. However, defeated mice treated with OXT did not show this increase in the neuroinflammatory response. In conclusion, OXT treatment can be a powerful therapeutic target to reduce the negative effects of social stress on ethanol consumption and the neuroinflammatory process.

1. Introduction

Oxytocin (OXT) is a nonapeptide produced in the supraoptic and paraventricular nuclei of the hypothalamus and is released into blood circulation through neurohypophysis. OXT not only acts as a modulating hormone of physiological functions such as uterine contractions and lactation (Leng et al., 2015; Russell et al., 2003), but also as a neuromodulator in the central nervous system (CNS), since it is involved in maternal (Kim and Strathearn, 2016; Marlin et al., 2015), reproductive (Veening et al., 2015) and social (Caldwell, 2017; Caldwell et al., 2017) behaviors. Clinical and preclinical studies have demonstrated that OXT has a modulating role in a wide variety of social interactions that have been relevant in the evolution of mammals, as in social recognition, sexual behavior, pairing, parental behavior and aggression (Ebert and Brüne, 2018; Chen et al., 2017; Lazzari et al., 2019; Lukas et al., 2011; Stohn et al., 2018). In addition, OXT has an anxiolytic effect and attenuates the hypothalamic-pituitary-adrenal (HPA) axis response to stress (Winter and Jurek, 2019; Yang et al., 2019). OXT also produces a reduction in amygdala functions and, therefore in stress reactivity (Kirsch et al., 2005; Labuschagne et al., 2010; Lukas et al., 2011; Nasanbuyan et al., 2018; Onaka et al., 2012). OXT produces an increase in the connectivity between the amygdala and the medial rostral prefrontal cortex, which plays a critical role in social cognition (Sripada et al., 2013). This action points to OXT as a therapeutic target in fear and social disorders such as post-traumatic stress disorder, generalized anxiety disorder or social anxiety disorder (Neumann and Landgraf, 2012; Steinman et al., 2019; Wang, L. et al., 2018).

In recent years, several studies have shown that administration of OXT reduces the effects caused by social stress, promoting an increase in social interaction and decreasing the anhedonia and social avoidance that characterizes social stress (Borland et al., 2018; Ebert and Brüne, 2018; Ferrer-Pérez et al., 2019; Lukas et al., 2011; Nasanbuyan et al., 2018; Steinman et al., 2016; Wang, L. et al., 2018). The most commonly used social stress model is social defeat stress (SD), based on the resident-intruder paradigm. This model evaluates avoidance/flee and defensive/submissive behaviors in experimental mice caused by the territoriality of aggressive residents (Covington III and Miczek, 2001; Miczek et al., 2004). Animal models of SD stress are known to produce

Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; CPP, conditioned place preference; DA, dopamine; EtOH, ethanol; FR1, fixed ratio 1; FR3, fixed ratio 3; HPA, hypothalamic-pituitary-adrenal axis; NAcc, nucleus accumbens; OXT, oxytocin; PR, progressive ratio; SA, self-administration; SD, social defeat; VTA, ventral tegmental area

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long-term consequences, such as anhedonia, decreased social interaction, anxiety, depression, or drug addiction (Burke and Miczek, 2014; Liu et al., 2017; Miczek et al., 2008; Shimamoto et al., 2015).

It is well known that social stress influences the development of addiction, increasing drug-seeking and taking behaviors, withdrawal and relapse (Koob and Schulkin, 2019; Montagud-Romero et al., 2018; Ruisoto and Contador, 2019). Rodents exposed to SD stress increase consumption, preference and motivation for ethanol (EtOH) in different paradigms, such as EtOH-induced conditioned place preference (CPP) (Karlsson et al., 2017; Macedo et al., 2018), the two-bottle choice (Croft et al., 2005; Deal et al., 2018; Newman et al., 2018) or drinking in the dark (Caruso et al., 2018). In oral self-administration (SA), our and other research groups described a significant increase of EtOH consumption and a greater motivation to obtain EtOH in socially defeated mice (Norman et al., 2015; Reguilón et al., 2020; Rodríguez-Arias et al., 2016; Van Erp and Miczek, 2001). It must also be noted that OXT administration decreases drug use, withdrawal symptoms, and drug-seeking behaviors associated with various drugs of abuse (Leong et al., 2018; Pedersen, 2017). Specifically, OXT administration reduces EtOH consumption in rodents (King et al., 2017; MacFadyen et al., 2016; Peters et al., 2017; Stevenson et al., 2017; Tunstall et al., 2019). The administration of OXT would normalize brain changes induced by drugs of abuse, thereby reducing consumption (King et al., 2020; Leong et al., 2018). Tunstall and co-workers (2019) proposed that OXT, by blocking GABA signaling at the central nucleus of the amygdala, decreases excessive alcohol consumption in alcohol-dependent rats. Other studies suggest that OXT acting on the mesolimbic pathway modifies dopaminergic signaling (Borland et al., 2018; Peris et al., 2017; Weber et al., 2018). In fact, subchronic administration of OXT decreases the basal release of dopamine (DA) in the nucleus accumbens (NAcc) (Estes et al., 2019). Additionally, intracerebroventricular OXT administration blocks the DA-release induced by the administration of EtOH in rats (Peters et al., 2017).

In the last decades, neuroinflammatory processes have been widely studied in addiction and stress-related disorders (Calcia et al., 2016; Northrop and Yamamoto, 2013; Orio et al., 2019; Zhang et al., 2018). After exposure to SD, there are reports of detriment in blood-brain barrier (BBB) permeability (Rodríguez-Arias et al., 2017) and an increase in cytokines (Ferrer-Pérez et al., 2018; Wohleb et al., 2011, 2012, 2014) and chemokines levels (Reguilón et al., 2020; Rossetti et al., 2016; Wohleb et al., 2013). Moreover, SD-induced neuroinflammation can exacerbate the well-known brain damage produced by EtOH (Karlsson et al., 2017; Montesinos et al., 2016; Pascual et al., 2015; Zhang et al., 2018). Interestingly, some recent studies have shown that OXT treatment mitigates the neuroinflammatory response in mice caused by maternal separation (Amini-Khoei et al., 2017) or post-traumatic stress (Wang, S. et al., 2018).

There are no studies evaluating the potential effect of OXT on the increased consumption of EtOH induced by social defeat. However, we know that mice treated with OXT prior to each SD did not develop the long-lasting increase in cocaine-induced CPP (Ferrer-Pérez et al., 2019). Therefore, in the present study we aimed to evaluate the effect of 1 mg/kg of OXT administered 30 min before each SD episode on the long-lasting increase in EtOH consumption and the neuroinflammatory response. After three weeks of the last SD encounter, we assessed the consumption and motivation for EtOH using the EtOH-SA oral paradigm. Furthermore, we also evaluated the levels of chemokines CX3CL1 and CXCL12 in the striatum after the last exposure to SD and at the end of the EtOH-SA procedure.

2. Material and methods

2.1. Subjects

105 male OF1 mice (Charles River, France) were delivered to our laboratory at 42 days of age. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998) (n = 15 adult mice). All mice were housed under the following conditions: constant temperature, a reversed light schedule (lights off at 08:00 and on at 20:00), and food and water were freely available ad libitum, except during the behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committee (University of Valencia).

2.2. Drugs

For the oral SA procedure, absolute EtOH (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) EtOH solution equivalent to a 7.6% (v/v) EtOH solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water. Before SD, mice were injected 1 mg/kg of oxytocin (Sigma-Aldrich, Madrid, Spain) in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs.

2.3. Experimental design

The experimental design is depicted in Fig. 1. The first set of mice, composed by 40 mice, were employed to evaluate the effect of OXT on the neuroinflammation response induced by SD. Half of the mice received an i.p. injection of saline or 1 mg/kg of OXT 30 min before each defeat procedure. Half of the mice in each group were sacrificed immediately after the first social defeat, while the remainder were sacrificed after the fourth defeat. The exploration group (CON) treated with saline 30 min before the procedure was sacrificed after the 1st exploration. The striatum was collected for further biochemical analysis.

The second set of mice was composed of a total of 50 mice that underwent an SD/CON protocol. Similarly, half of the mice received an i.p. injection of saline or 1 mg/kg of oxytocin 30 min before each exploration or defeat procedure. Subsequently, three weeks after the last defeat, the mice initiated the EtOH SA protocol for approximately 28 days. At the end of this test, all the mice were sacrificed to obtain the striatum for further analysis of the chemokine levels (8 mice of each experimental group).

2.4. Repeated social defeat encounters

Animals in the stress/defeated groups were exposed to 4 episodes of SD lasting 25 min each on postnatal days (PND) 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the aggressive resident (Covington III and Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each social defeat protocol was video-recorded and ethologically analysed. Threat and attack behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was replaced for a further 10 min to allow social threats from the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, after which the behavioral tests began.

In the corresponding groups, physiological saline or oxytocin was administered 30 min before each social encounter or exploration (control groups).

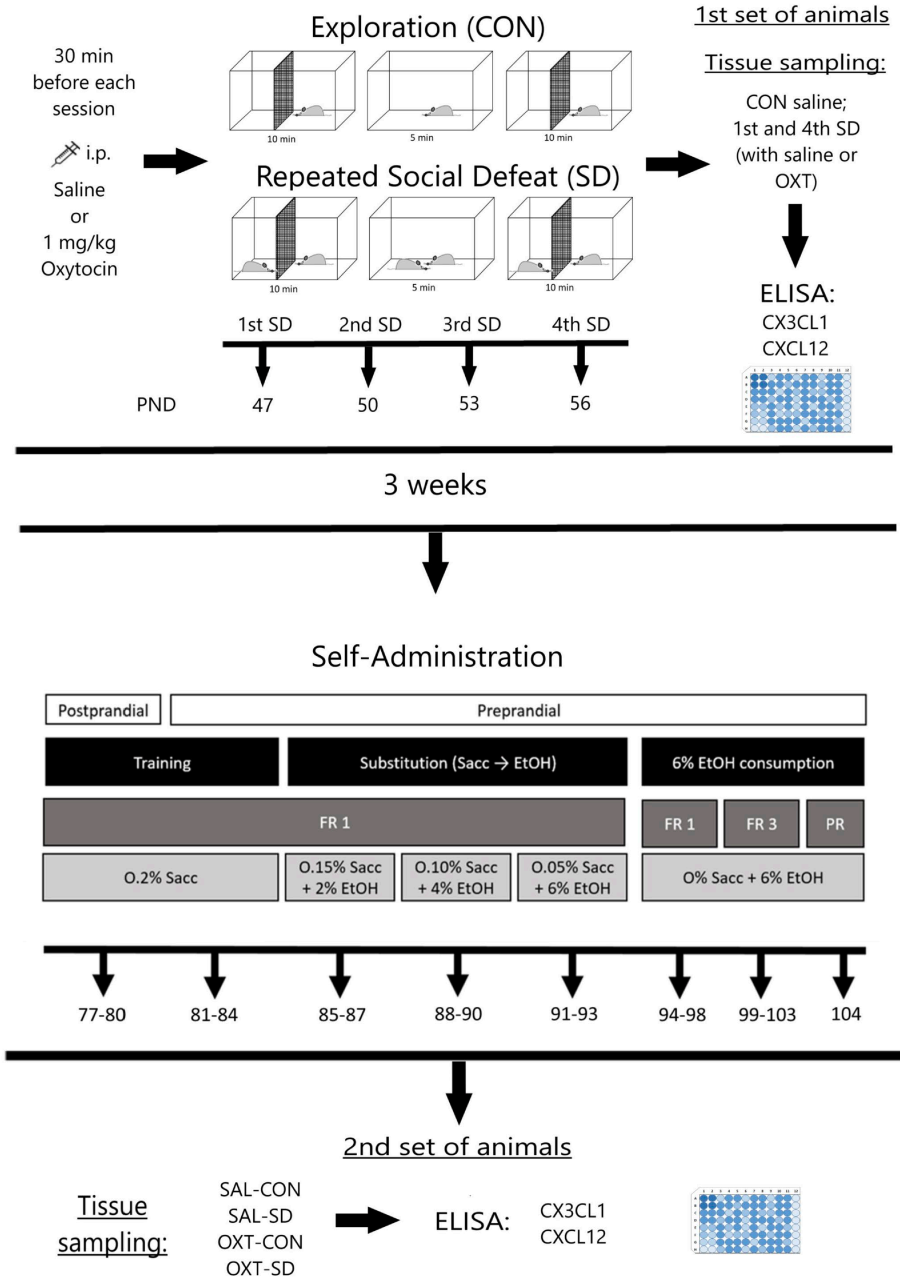

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Fig. 1. Experimental design. All the mice were divided into four experimental groups: SAL-CON, SAL-SD, OXT-CON and OXT-SD. Saline or 1 mg / kg OXT were injected 30 min before each exposure to exploration (CON) or SD. Four sessions were carried out every 72 h of SD or exploration in the corresponding groups. A 1st set of mice was sacrificed to carry out the biochemical analyses 4 h after the 1st exposure to the exploration, 8 mice were sacrificed and, in the groups exposed to stress, the sacrifice and extraction of samples were performed 4 h after the 1st and 4th exposures to SD (8 mice per group). After finishing the encounters, the rest of the mice were kept without manipulation for 3 weeks, and then the protocol of oral EtOH SA began. This procedure lasted 28 calendar days and 24 h after the end of the PR schedule, the mice were sacrificed to obtain the samples for their subsequent biochemical analysis (2nd set of mice).

2.5. Apparatus and procedures: oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral EtOH SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-poke delivered 36 μl of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6 s time-out period. The inactive nose-poke did not produce any consequence.

To evaluate the consequences of SD (with or without oxytocin) on the acquisition of oral EtOH SA, animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% EtOH consumption.

2.5.1. Training phase (8 days)

Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and food allotment was provided 1 h prior to the session to increase the motivation for active nose-poking. During the subsequent 3 days, water was provided ad libitum, except during the 1 h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1 h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 μl of 0.2% (w/v) saccharin reinforcement.

2.5.2. Saccharin substitution (9 days)

The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac − 2% EtOH; 0.10% Sac − 4% EtOH; 0.05% Sac − 6% EtOH).

2.5.3. 6% ethanol consumption (11 days)

The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 6% EtOH (w/v) intake and the motivation to drink. This phase began 38 days after the last social defeat. After each session, the alcohol that remains in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of effective responses and EtOH consumption (μl) were measured under fixed ratio 1 (FR1) for 5 daily consecutive sessions, fixed ratio 3 (FR3) (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement, according to the previous scale (for example, if an animal activates the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement). Therefore, the breaking point value for this animal would be 40. All the sessions lasted 1 h, except the PR session, which lasted 2 h.

2.6. Tissue sampling

Striatum samples were taken 4 h after the first exploration, the first and the fourth agonistic encounters and a final sample was obtained after SA.

To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and the striatum dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Paxinos and Franklin, 2001), which were then kept in dry ice until storage at −80 °C. Before CX3CL1 and CXCL12 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al., 2010. Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM $Na3VO4$, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

2.7. Determination of CX3CL1 and CXCL12 levels

To determine CX3CL1 and CXCL12 concentration on tissues, we used a Mouse CX3CL1 ELISA Kit obtained from Abcam (Ref: ab100683) and a Mouse CXCL12 Kit obtained from Abcam (Ref: ab100741), which were performed following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450 nm and final results were calculated using a standard curve following the manufacturer's instructions, and were expressed as ng/mg for CX3CL1 and as pg/mg for CXCL12 (tissues).

2.8. Statistical analysis

The data of the ethological analysis of resident and intruder mice were analysed by a two-way ANOVA with a one between-subjects variables – Treatment (with or without OXT)- and a one within variable - SD encounter- with two levels: first and fourth SD.

To analyse acquisition of EtOH SA, a three-way ANOVA was performed with two between-subjects variables –Stress (CON or SD) and Treatment (saline or OXT)– and a within-subjects variable in both cases –Days, with five levels of FR1 or FR3–. The effects of SD and treatment on breaking point values and EtOH consumption during PR was analysed by a two-way ANOVA, with two between-subjects variable –Stress (CON or SD) and Treatment (Saline and OXT).

Data related to chemokine concentrations after SA procedure were analysed using a two-way ANOVA, with two between-subjects variables –Stress (CON or SD) and Treatment (Saline and OXT). For the striatal chemokine concentrations after the SD, a one-way ANOVA was

Table 1

Ethological analyses of social defeat. Behavior of resident and intruder mice during 5-min agonistic encounters. p < ***, p < **, p < * significant differences between the 1st and 4th SDs. p < + + +, p < + +, p < + significant differences between SAL-treated and OXT-treated mice.

		Encounters	Treated with saline		Treated with oxytocin	
			1st	4th	1st	4th
Intruder mice	Avoidance/flee	Total time (s)	21.6 ± 2	16.3 ± 1.5**	27.2 ± 5.6	15.7 ± 1.8**
		Mean time (s)	1.9 ± 0.4	0.9 ± 0.1***	2.2 ± 0.3	1 ± 0.1***
		Latency	30 ± 7.2	6.6 ± 2.1	10.9 ± 6.1	11.5 ± 9.1
	Defence/submission	Total time (s)	21 ± 3.2	40 ± 2**	25.1 ± 7.3	42.6 ± 5.2**
		Mean time (s)	1.8 ± 0.4	1.7 ± 0.1	1.7 ± 0.4	2.1 ± 0.1
		Latency	48.4 ± 12.3	13.1 ± 4.8*	74.1 ± 40.5	3.4 ± 1.7*
Resident mice	Threat	Total time (s)	13.2 ± 1.3	9 ± 0.6*	7 ± 0.7^{+++}	7.4 ± 1.5
		Mean time (s)	1.4 ± 0.2	1.1 ± 0.1	1 ± 0.1^{+}	0.8 ± 0.1^{+}
		Latency	28.5 ± 5.9	9 ± 0.6*	4.2 ± 1.4^{+++}	14.3 ± 9.2
	Attack	Total time (s)	9.8 ± 0.7	28.9 ± 3***	10.9 ± 6.2	30.5 ± 3.2***
		Mean time (s)	0.9 ± 0.1	2 ± 0.2*	1.4 ± 0.9	2.3 ± 0.1*
		Latency	49.5 ± 14.2	15.7 ± 5.2***	3.3 ± 0.8^{++}	2.7 ± 1.2^{+}

employed with a between variable –Group (CON, 1st SAL-SD, 4th SAL-SD, 1st OXT-SD, 4th OXT-SD).

All ANOVAs were followed by a Bonferroni's post-hoc test and partial eta-square (η_p^2) was performed to calculate effect sizes. Moreover, Cohen's d effect sizes were calculated for all statistically different pair-wise comparisons. The results are reported as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. *OXT treatment does not affect the ethological behavior of repeated SD*

With regard to intruder mice, the ANOVA revealed a significant difference in the variables Day for Total Time [$F(1, 18) = 8.246$; $p = 0.01$; $\eta_p^2 = 0.314$] and Day for Mean Time [$F(1, 18) = 15.179$; $p = 0.001$; $\eta_p^2 = 0.457$] in Avoidance/Flee behavior. All mice spent less total ($p = 0.01$, $d = 0.8880$) and mean ($p = 0.001$, $d = 1.3916$) time in Avoidance/Flee behavior. Moreover, the ANOVA revealed a significant difference in the variables Day for Total Time [$F(1, 18) = 9.784$; $p < 0.01$; $\eta_p^2 = 0.352$] and Day for Latency [$F(1, 18) = 6.799$; $p < 0.05$; $\eta_p^2 = 0.274$] in Submission/Defensive behavior (Table 1). All mice spent more time in Submission/Defensive behavior in the fourth SD compared with the first SD ($p < 0.01$, $d = 1.2751$,) and showed a higher latency of this behavior in the first SD compared to the fourth SD ($p < 0.05$, $d = 0.8415$).

In the behavior of the resident mice, the ANOVA revealed a significant difference in the variable Treatment for Mean Time [$F(1, 18) = 6.211$; $p < 0.05$; $\eta_p^2 = 0.257$] and in the interactions Day × Treatment for Total Time [$F(1, 18) = 4.731$; $p < 0.05$; $\eta_p^2 = 0.208$], and Day × Treatment for Latency [$F(1, 18) = 5.925$; $p < 0.05$; $\eta_p^2 = 0.248$] in Threat behavior (Table 1). The post-hoc comparison showed that resident mice spent less mean time threating OXT-treated mice ($p < 0.05$, $d = 0.8645$), and resident mice spent less time threating OXT-treated mice during the first SD ($p < 0.001$, $d = 3.8923$), but no difference was observed in the last SD. In addition, resident mice showed more threat behavior to saline-treated mice during the first SD than the fourth ($p < 0.05$, $d = 3.04539$). Finally, resident mice showed a higher latency of threat from SAL-treated mice in the first SD compared to fourth SD ($p < 0.05$, $d = 1.1711$) and showed a higher latency of threat from SAL-treated mice in the first SD compared to OXT-treated mice ($p = 0.001$, $d = 1.8749$). Regarding attack behavior, the ANOVA showed an effect of the variables Day for Total Time [$F(1, 18) = 32.331$; $p < 0.001$; $\eta_p^2 = 0.642$] and Day for Mean Time [$F(1, 18) = 5.872$; $p < 0.05$; $\eta_p^2 = 0.246$], and in the interaction Day × Treatment for Latency [$F(1, 18) = 5.925$; $p = 0.01$; $\eta_p^2 = 0.315$]. Resident mice showed more total ($p < 0.001$, $d = 1.74268$) and mean ($p < 0.05$, $d = 0.7357$) time in attack behavior in the last SD compared to the first. On the one hand, resident mice showed a higher latency of attack behavior with the SAL-treated mice during the first SD compared to the fourth SD ($p = 0.001$, $d = 0.9259$). On the other hand, they showed a higher latency of attack behavior with SAL-treated mice compared to OXT-treated mice during the first ($p < 0.01$, $d = 1.3989$) and fourth ($p < 0.05$, $d = 1.1589$) SDs.

3.2. *Oxytocin attenuates the increase in oral ethanol SA induced by social stress*

No differences were found between the animals during training or substitution phases, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of effective responses during the FR1 schedule of EtOH SA revealed a significant effect of the interaction Days × Treatment [$F(4, 184) = 3.488$; $p < 0.01$; $\eta_p^2 = 0.070$] and Stress × Treatment [$F(1, 46) = 6.301$; $p < 0.05$; $\eta_p^2 = 0.120$] (Fig. 2a). Post-hoc comparison showed that mice treated with oxytocin showed fewer effective responses compared to non-treated mice on days 1 ($p < 0.05$, $d = 0.68580$), 2 ($p < 0.001$, $d = 1.17522$) and 3 ($p < 0.01$, $d = 1.05017$). Moreover, effective responses were higher in the saline-treated defeated group (SAL-SD) with respect to the rest of the groups (p's < 0.001 in all cases; $d = 2.248$ for SAL-CON group, $d = 2.542$ for OXT-CON group, $d = 1.487$ for OXT-SD group). With respect to EtOH consumption, the ANOVA revealed a significant effect of the interaction Stress × Treatment ([$F(1, 46) = 12.678$; $p = 0.001$; $\eta_p^2 = 0.217$]) (Fig. 2b). The post-hoc comparison showed that the SAL-SD group consumed EtOH at higher rates than the rest of the groups (p's < 0.001 in all cases; $d = 1.7045$ for SAL-CON group, $d = 1.7697$ for OXT-CON group, $d = 1.1609$ for OXT-SD group).

During the FR3 schedule, the ANOVA revealed a significant effect of the variable Stress [$F(1, 46) = 6.866$; $p < 0.05$; $\eta_p^2 = 0.130$] and the interaction Days × Treatment [$F(4, 184) = 4.940$; $p = 0.001$; $\eta_p^2 = 0.097$] for the number of effective responses (Fig. 2a). Defeated groups (SAL-SD and OXT-SD) showed higher number of effective responses than non-stressed groups ($p < 0.05$; $d = 0.664$). Moreover, mice treated with oxytocin (OXT-CON and OXT-SD) showed a lower number of effective responses on day 8 compared to day 9 ($p = 0.0$; $d = 0.507$) and saline-treated groups (SAL-CON and SAL-SD) showed a lower number of effective responses on day 6 compared to days 7 ($p < 0.01$; $d = 0.576$) and 8 ($p < 0.05$; $d = 0.509$). With respect to EtOH consumption, the ANOVA revealed a significant effect of the variable Stress [$F(1, 46) = 5.084$; $p < 0.05$; $\eta_p^2 = 0.100$] and the interaction Days × Treatment [$F(4, 184) = 6.308$; $p < 0.001$; $\eta_p^2 = 0.121$] (Fig. 2b). Defeated groups (SAL-SD and OXT-SD) consumed significantly more EtOH than non-stressed groups ($p < 0.05$;

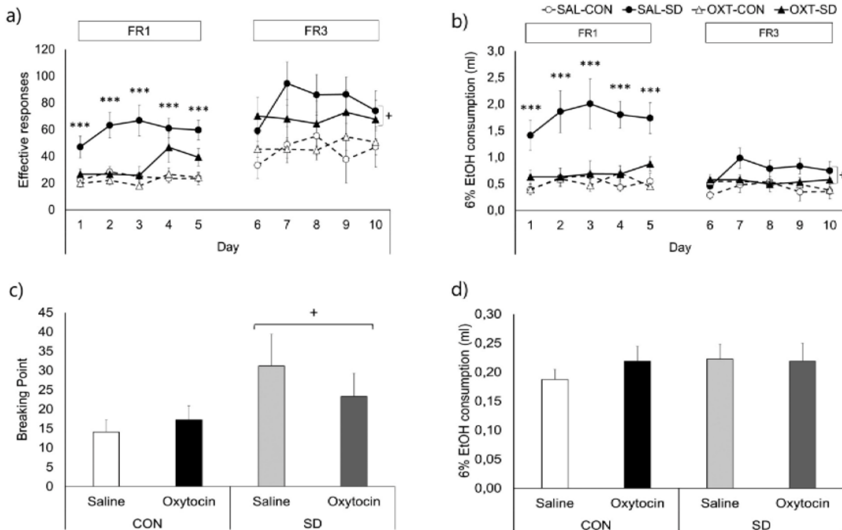

Fig. 2. Effects of oxytocin treatment during SD procedure on the increase in oral EtOH self-administration induced by social stress in OF1 mice. Mice were divided in the following four treatment groups: CON mice were allowed to explore a new cage and were treated with saline (SAL-CON, n = 14) or with oxytocin (OXT-CON, n = 12); The SD group was exposed to social defeat and treated with saline (SAL-SD, n = 12) or with oxytocin (OXT-SD, n = 12). The dots represent means and the vertical lines ± SEM of (a) the number of effective responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent means and the vertical lines ± SEM of (c) the breaking point values and (d) the volume of 6% EtOH consumption during PR. ***p < 0.001 significant difference with SAL-CON, OXT-CON and OXT-SD; + p < 0.05 significant difference with defeated groups (SAL-SD, OXT-SD) with respect to non-defeated groups (SAL-CON, OXT-CON).

$d = 0.5476$). Also, all animals treated with oxytocin (OXT-CON and OXT-SD) showed significantly higher consumption rates on day 6 ($p < 0.05$; $d = 0.666$) and a significantly lower consumption rate on day 8 ($p < 0.05$; $d = 0.644$) with respect to saline-treated groups.

During the PR, the ANOVA for the breaking point values of EtOH SA revealed a significant effect of the variable Stress [$F(1, 46) = 4.769$; $p < 0.05$; $\eta_p^2 = 0.094$] (Fig. 2c). The post-hoc comparison showed that the breaking point values were higher in the defeated groups (SAL-SD and OXT-SD) with respect to the non-stressed groups ($p < 0.05$; $d = 0.6227$). The ANOVA for EtOH consumption during PR did not reveal any significant effect (Fig. 2d).

3.3. OXT blocked the increase in CX3CL1 and CXCL12 striatal levels induced by SD

The ANOVA revealed a significant effect of the variable Group [$F(4, 35) = 9.055$; $p < 0.001$; $\eta_p^2 = 0.509$] for the striatal levels of CX3CL1 (Fig. 3a). The post-hoc comparison showed that defeated animals treated with saline (SAL-SD) showed higher CX3CL1 levels after the 4th defeat than those of the exploration condition (SAL-CON) ($p < 0.001$; $d = 1.8313$). In addition, after the 4th SD encounter, the SAL-SD group showed a significantly higher striatal level of CX3CL1 than the OXT-SD group ($p < 0.001$; $d = 2.1480$).

With respect to the striatal protein levels of CXCL12, the ANOVA showed a significant effect of the variable Group [$F(4, 35) = 11.874$; $p < 0.001$; $\eta_p^2 = 0.576$] (Fig. 3b). The post-hoc comparison showed that the SAL-SD group showed higher CXCL12 levels after the 4th defeat compared to the SAL-CON group ($p < 0.01$; $d = 1.997$). After the 1st and 4th SD encounters, the SAL-SD group showed higher CXCL12 levels than the OXT-SD group ($p < 0.05$; $d = 1.551$ and $p < 0.001$; $d = 2.979$, respectively).

3.4. OXT blocked the increase in CX3CL1 and CXCL12 striatal levels observed in defeated mice after oral ethanol SA

The ANOVA for CX3CL1 and CXCL12 striatal levels showed a significant effect of the interaction Stress × Treatment [$F(1, 28) = 20.019$; $p < 0.001$; $\eta_p^2 = 0.417$] (Fig. 4a) and [$F(1, 28) = 4.686$; $p < 0.05$; $\eta_p^2 = 0.143$] (Fig. 4b). The post-hoc comparison revealed that defeated animals treated with saline (SAL-SD) showed higher concentrations of these chemokines compared to the rest of the groups (p's < 0.001; $d = 2.845$ in SAL-CON group, $d = 3.051$ in OXT-CON group and $d = 3.317$ in OXT-SD group for CX3CL1 and p's < 0.01; $d = 2.160$ in SAL-CON group, $d = 1.584$ in OXT-CON group and $d = 1.351$ in OXT-SD group for CXCL12).

4. Discussion

Our results showed for the first time that OXT administration before each SD blocked the increase in EtOH intake induced by SD. OXT treatment also reduced the neuroinflammation response measured by the striatal levels of the chemokines CX3CL1 and CXCL12 immediately after SD and at the end of the oral EtOH-SA. However, OXT treatment failed to reduce the motivation to obtain EtOH in the PR schedule.

As OXT was administered prior to each SD, it was critical to corroborate that the behavior of the intruder and the resident mice did not vary due to the OXT administration. As we have previously shown (Ferrer-Pérez et al., 2019), treatment with OXT prior to each SD does not alter the behavior of the intruder mice. OXT-treated mice spent similar amounts of time in avoidance/flee and defence/submission behaviors as SAL-treated mice. During the first defeat, resident mice threatened OXT-treated intruders for a shorter time compared to the saline group, differences that disappeared in the last SD. In addition, no

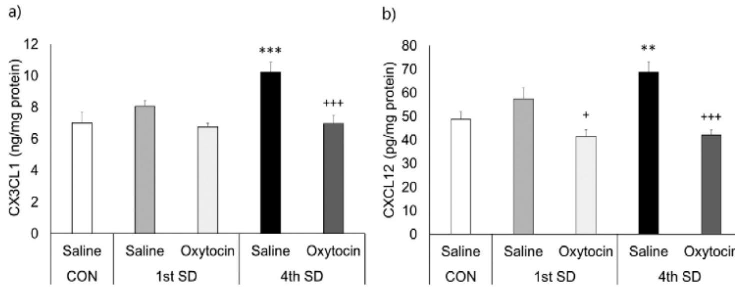

Fig. 3. Striatal levels of CX3CL1 and CXCL12 after SD. Concentration of (a) CX3CL1 and (b) CXCL12 after exploration condition (CON), 1st defeat in mice treated with saline (1st SAL-SD) or oxytocin (1st OXT-SD) and 4th defeat in mice treated with saline (4th SAL-SD) or oxytocin (4th OXT-SD). The columns represent means and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice (n = 8 in all groups). $^{***}p < 0.001$, $^{**}p < 0.01$ differences with respect to the SAL-CON group; $+++p < 0.001$, $+p < 0.05$ differences with respect the corresponding SAL-SD group.

differences in attack behavior were observed depending on the treatment. Although SD was effectively experienced by animals treated with OXT, we cannot rule out differences in the way OXT-treated animals coped with the SD experience.

The current study corroborates that SD produces a long-lasting increase in EtOH intake using the oral EtOH-SA paradigm (Caldwell and Riccio, 2010; Norman et al., 2015; Reguilón et al., 2020; Rodríguez-Arias et al., 2016). In the present study, we observed that defeated SAL-treated mice consumed more EtOH and showed a higher number of effective responses compared to SAL-treated, non-stressed animals during the FR1 schedule. Conversely, defeated OXT-treated mice consumed significantly less EtOH and showed fewer effective responses. To our knowledge, there are no studies evaluating the role of OXT on the increase in the oral EtOH-SA paradigm induced by SD. Several studies have established that OXT treatment reduces EtOH consumption (King et al., 2017; MacFadyen et al., 2016; Peters et al., 2017), and operant responses in rodents (Tunstall et al., 2019). These results suggest that OXT can modulate the DA pathway projecting from the hypothalamus onto the ventral tegmental area (VTA) and the NAcc, which present OXT receptors (Grinevich et al., 2016). OXT targets VTA DA neurons, increasing extracellular DA in the NAcc, thereby increasing positive enhancements and signaling of natural rewards (Adinoff, 2004; Hung et al., 2017). Therefore, OXT stimulation in DA reward circuits can decrease EtOH-induced stimulation of DA neurons (Peris et al., 2020). However, in our experimental design, OXT was administered during SD, weeks earlier than the beginning of the EtOH exposure. Therefore, these direct mechanisms of OXT cannot explain our results. Moreover, OXT can potentially reduce corticosterone levels and normalize the HPA axis stress response (Peris et al., 2020). King and Becker (2019) observed that OXT attenuated the EtOH-seeking and reinstatement behavior caused by immediate stress (15 min before SA session).

The anxiolytic and anti-stress effects of OXT have been widely demonstrated (Bülbül et al., 2011; Grund et al., 2019; Krause et al., 2011; Peters et al., 2014; Smith et al., 2015). In this way, OXT could interfere in the experience of SD, decreasing the negative impact of stress. Supporting this hypothesis, we observed that a similar OXT administration than the one employed in this study can decrease the long-lasting increase in anxiety observed in socially defeated animals (Ferrer-Pérez et al., 2019). There is lack of consensus in the scientific literature about the effects of OXT on anxiety and stress. OXT can induce anxiolytic or anxiogenic effects modulating the salience of emotional contexts (see Jurek and Meyer, 2020), although the mechanisms are unclear (Shamay-Tsoory and Abu-Akel, 2016). The results observed vary considerably according to the dose used and the method of administration. Chronic OXT treatment increases the HPA response (Yoon and Kim, 2020), but acute administration only transiently increases corticosterone levels (Petersson et al., 1999). Intracerebroventricular infusions of low doses of OXT reduce the effects of chronic stress, but high doses increase the anxiogenic behavior (Peters et al., 2014). However, infusions of OXT in the medial prefrontal cortex increase dopaminergic transmission, inducing an antidepressant effect in animals previously subjected to SD (Li et al., 2020). Important sex differences in the OXT

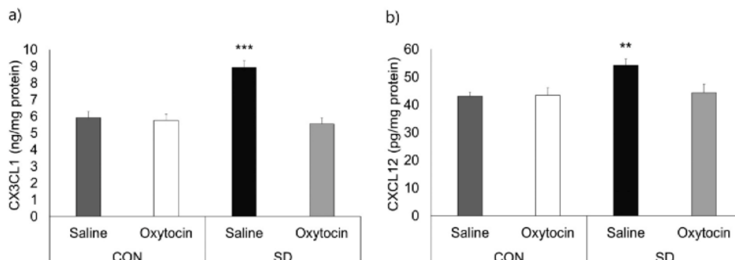

Fig. 4. Striatal levels of CX3CL1 and CXCL12 after EtOH SA. CX3CL1 (a) and CXCL12 (b) protein levels after oral EtOH SA in the following treatment groups: CON group allowed to explore a new cage and treated with saline (SAL-CON, n = 8) or with oxytocin (OXT-CON, n = 8); and SD group exposed to social defeat and treated with saline (SAL-SD, n = 8) or with oxytocin (OXT-SD, n = 8). The columns represent means and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice. $^{***}p < 0.001$; $^{**}p < 0.00$ significant differences with the rest of the groups.

modulation of stress effects have also been observed. While administration of OXT before SD produces an increase in the anxiogenic effects in females, a decrease is observed in males (Steinman et al., 2016; Steinman and Trainor, 2017).

Regarding the FR3 and PR schedules of the SA paradigm, a stress effect was obtained independent of OXT treatment. SAL- and OXT-treated defeated mice showed a greater effort to obtain a dose of EtOH during the FR3 schedule and showed a significantly higher breaking point during the PR schedule compared to non-stressed animals. The FR1 procedure assesses the potential liability of a drug and the consumption based on its unconditioned psychopharmacological effects (Sanchis-Segura and Spanagel, 2006). However, FR1 performance is less affected by incentive value or motivational factors. The activities of some neural systems (Salamone and Correa, 2002) or experimental conditions (Morgan et al., 2002) do not modify the number of reinforcers obtained under an FR1 schedule, resulting in significant behavioral changes when using progressive increases in ratio. Some studies associate an increased motivation (breaking point) during PR to DA release from the dorsolateral striatum (González-Marín et al., 2019). Dorsolateral striatum is involved in both the rewarding and motor effects of EtOH and specific aspects of incentive motivation (Chen et al., 2015), the signals that activate incentive salience in instrumental learning seem to be associated with DA activity in the dorsolateral striatum (González-Marín et al., 2019; Ostlund et al., 2011). Since the VTA dopaminergic system is extremely sensitive to stress (Anstrom et al., 2009; Krishnan et al., 2008; Miczek et al., 2008; Razzoli et al., 2011), one possible explanation could be that the OXT dose administered was incapable of blocking the increased motivation for EtOH induced by SD. Although many studies claim that OXT administration reduces motivation in oral EtOH-SA (King et al., 2017; Tunstall et al., 2019), in these studies OXT is administrated immediately prior to the EtOH-SA paradigm.

SD induced an immediate and long-lasting increase in the striatal levels of both chemokines, CXC3L1 and CXCL12. Fractalkine or CX3CL1 has both an inflammatory and a proinflammatory function, and promotes microglial and astrocytic activation, proinflammatory cytokine secretion, ICAM-1 expression, and CNS T-cell recruitment during neuroinflammatory diseases (Galán-Ganga et al., 2019; Lauro et al., 2015; Lee et al., 2018). CXCL12 promotes the growth of neurites and neurogenesis (Jaerve and Müller, 2012; Opatz et al., 2009) but is also involved in cell migration that contributes to inflammation, attracting leukocytes through the BBB. Nevertheless, excessive production and accumulation of CXCL12 can be toxic and this inflammation can lead to brain damage. In the present study, we observed that the concentration level of both chemokines is higher after the 4th SD in defeated mice compared to non-stressed mice. Furthermore, we observed an anti-inflammatory effect of OXT, since defeated mice treated with OXT showed significantly lower levels of CX3CL1 after the first and fourth SD compared to the SAL-treated defeated mice. Similar results were observed with the striatal levels of CXCL12.

We have previously shown that SD induced a long-lasting increase in brain cytokines, as IL-6, (Ferrer-Pérez et al., 2018) and chemokines, as CX3CL1 and CXCL12, (Reguilón et al., 2020). Our results confirmed these findings and, more importantly, showed that prior administration of OXT blocked this neuroinflammatory response. There are no previous studies linking OXT administration and the neuroinflammatory response caused by SD. OXT has a short-term anti-inflammatory effect on the innate immune response (Bordt et al., 2019; Yuan et al., 2016), suppressing TNF-α, IL-1β, COX-2, and iNOS expression caused by injection of liposaccharide bacteria (Inoue et al., 2019; Yuan et al., 2016). The protective effects of OXT have also been shown in rodents with brain damage or infection in which OXT induced an immediate decrease in the circulatory levels of proinflammatory cytokines and the infiltration of neutrophils in injured areas (Inoue et al., 2019; Yuan et al., 2016).

Moreover, these results were confirmed after the end of the EtOH-SA paradigm. Again, defeated mice showed higher levels of both chemokines when compared to non-stressed animals. Although exposure to EtOH is an important promoter of neuroinflammation (Pradier et al., 2018), we only observed a neuroinflammatory response after EtOH SA in the defeated group treated with saline. Saline non-stressed mice showed comparable levels of CX3CL1 and CXCL12 to those of the exploration group prior to EtOH SA exposure, non-exposed to stress or EtOH. This may be due to the fact that the concentration of EtOH used in SA is low (6%) and that the daily intake of non-stressed mice was not enough to induce neuroinflammation. On the other hand, defeated mice had higher EtOH intake and an elevated neuroinflammation response after SD, which in turn induced an increase of CX3CL1 and CXCL12 striatal levels. Stressed mice treated with OXT showed a significantly lower concentration of both chemokines than defeated mice treated with saline. This could be due to the same anti-inflammatory effect already shown after SD, but we have to bear in mind that, after SA, OXT-defeated mice consumed an amount of EtOH comparable to the amount consumed by saline non-stressed animals. Therefore, we have to take into consideration that the lack of neuroinflammatory response could be due to the lower EtOH intake.

5. Conclusions

In conclusion, our results suggest that OXT decreases the SD-induced increase in EtOH consumption. This effect could be due to a decrease in the neuroinflammatory response induced by SD, although other mechanisms cannot be ruled out, such as a different coping with the defeat experience by the OXT-treated animals. These results point to OXT as a therapeutic target to reduce the negative effects of social stress on EtOH consumption and the neuroinflammatory process.

Data availability

The datasets generated for this study are available on request to the corresponding author.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Annex 2: Study 2. Voluntary wheel running protects against the increase in ethanol consumption induced by social stress in mice

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Voluntary wheel running protects against the increase in ethanol consumption induced by social stress in mice

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A B S T R A C T

Previous studies have shown that exposure to social defeat (SD), a model of social stress, produces a long-term increase in the consumption of ethanol, most likely through an increase in the neuroinflammation response. The aim of the present study was to evaluate whether exposure to physical activity in the form of voluntary wheel running (VWR) could block the increase in ethanol consumption and the neuroinflammatory response induced by social stress. Mice were exposed to either 4 sessions of repeated social defeat (RSD) or a non-stressful experience. During the whole procedure, half of the mice were exposed to controlled physical activity, being allowed 1 h access to a low-profile running wheel three times a week. Three weeks after the last RSD, animals started the oral self-administration (SA) of ethanol (6% EtOH) procedure. Biological samples were taken 4 h after the first and the fourth RSD, 3 weeks after the last RSD, and after the SA procedure. Brain tissue (striatum) was used to determine protein levels of the chemokines fractalkine (CX3CL1) and SDF-1 (CXCL12). RSD induced an increase in ethanol consumption and caused greater motivation to obtain ethanol. The striatal levels of CX3CL1 and CXCL12 were also increased after the last RSD. VWR was able to reverse the increase in ethanol intake induced by social stress and the neuroinflammatory response. In conclusion, our results suggest that VWR could be a promising tool to prevent and reduce the detrimental effects induced by social stress.

1. Introduction

Social stress is deeply implicated in the neural and behavioral alterations that contribute to the development of mental health disturbances and drug addiction (Beutel et al., 2018). Stressful experiences modify the reward system and are involved in the transition from drug abuse to addiction, causing an increase of intake and drug-seeking behaviors (Koob and Schulkin, 2019; Miczek et al., 2008; Montagud-Romero et al., 2016, 2018; Ruisoto and Contador, 2019). Social defeat (SD) is one of the most commonly used animal models to study the effects of stressful experiences. In this model, the experimental subject is repeatedly confronted with an aggressive opponent mouse (Miczek et al., 2004). SD induce a short-term increase in consumption of ethanol (EtOH) using oral self-administration (SA) with a higher motivation to get the drug (Van Erp and Miczek, 2001; Norman et al., 2015). In a previous study, we found that the effects of repeated social defeat (RSD) can be long-lasting, since mice exposed to RSD during adolescence showed higher ethanol consumption rates and a greater motivation to get the drug during adulthood (Rodríguez-Arias et al., 2016). Even after 6 months since the last stress exposure, defeated animals showed an enhanced motivation for ethanol intake (Riga et al., 2014). In addition, many studies support that social stress is one of the most important factors that influence the increase and escalation in ethanol consumption. Using voluntary ethanol intake, in the two bottle choice (TBC) task, SD produces an escalation in the consumption of alcohol after 10 days since the last exposure of stress (Norman et al., 2015; Hwa et al., 2016; Karlsson et al., 2017; Newman et al., 2018), although this effect is not observed immediately after being exposed to stress (Lopez et al., 2016). This increase in ethanol consumption induced by social stress could be due to stress-induced neuroadaptations, which ultimately produce changes in the hypothalamic, extrahypothalamic and mesocorticolimbic circuits, which are related to stress and reward (Holly et al., 2016; Hwa et al., 2016; Laine et al., 2017; Newman et al., 2018).

Nowadays, physical activity has emerged as a modulator of higher mental functions. Voluntary wheel running (VWR) in rodents produces enhanced learning, neurogenesis, angiogenesis, increases in

Abbreviations: RSD, repeated social defeat; SD, social defeat; EtOH, ethanol; SA, self-administration; FR1, fixed ratio 1; FR3, fixed ratio 3; PR, progressive ratio; TBC, two bottle choice; VWR, voluntary wheel running; HPA, hypothalamic-pituitary-adrenal; CPP, conditioned place preference; BBB, blood-brain barrier; PND, postnatal day

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neurotrophic factors and changes in several signaling molecules, as well as a reduction in behaviors associated with stress (Salam et al., 2009; Mul, 2018). VWR exercise after SD reduced social avoidance and anhedonia in rodents (Mul et al., 2018; Watanasriyakul et al., 2018; Zhang et al., 2019). It is known that physical exercise regulates some components of the hypothalamic-pituitary-adrenal axis (HPA), generating an adaptive response to stress (Pietrelli et al., 2018). Moreover, rats exposed to long-term access to VWR showed alterations in gene transcription factors involved in reward and dopaminergic neurotransmission in the mesolimbic reward pathway, developing conditioned place preference (CPP) to the compartment associated with physical exercise (Greenwood et al., 2011). Therefore, mice consumed significantly less ethanol in the unlimited access TBC model when they had access to the wheel (Ehringer et al., 2009; Darlington et al., 2014, 2016).

A number of recent reports have studied the relationship between stress, addiction and the immune system. Both exposure to stress and ethanol consumption activate the immune system and induce neuroinflammation (Calcia et al., 2016; Finnell and Wood, 2016; Rodríguez-Arias et al., 2017; Ferrer-Pérez et al., 2018; Montagud-Romero et al., 2018). Moreover, deregulation in chemokine signaling and neuroinflammation have been proposed to contribute to cognitive dysfunction and mental illness (Keogh and Parker, 2011; Wohleb et al., 2013; Pascual et al., 2015). SD-induced neuroinflammation has been clearly demonstrated, characterized by an activation of microglia (Stankiewicz et al., 2015), an increase of pro-inflammatory cytokines (Wohleb et al., 2011, 2012, 2014; Ferrer-Pérez et al., 2018), or the cross of peripheral immune cells to the CNS due to higher blood-brain barrier (BBB) permeability (Rodríguez-Arias et al., 2017).

There are no current studies evaluating the role of VWR in ameliorating the increase in EtOH consumption induced by SD. In mice and humans, several studies suggest that excessive or forced physical exercise produces brain injury and neuroinflammation (Svensson et al., 2016; Paolucci et al., 2018). However, physical exercise also upregulates tight-junction associated proteins of the BBB and protects the brain from injury, reducing the activation of microglia and cytokine levels in the hippocampus in mice (Park et al., 2016; Spielman et al., 2017) and humans (Paolucci et al., 2018). Therefore, it is necessary to evaluate if the neuroinflammatory process induced by RSD mediates the increase in EtOH consumption and if VWR could modify it. The aim of the present study was, firstly, to confirm that RSD induces a long-lasting increase in EtOH consumption using oral EtOH SA when experienced during adulthood; secondly, to evaluate if VWR could decrease these RSD effects on EtOH; and finally, to evaluate the neuroinflammatory response induced by RSD and EtOH, measuring the striatal levels of two chemokines fractalkine (CX3CL1) and SDF-1 (CXCL12). Chemokines are a family of small cytokines with chemo-attraction characteristics. Social stress is known to intervene in the signaling of chemokines on microglial morpho-functional activity (Wohleb et al., 2013; Sawicki et al., 2015; Milior et al., 2016) and, in addition, the striatal levels CX3CL1 increase after EtOH intake (Pascual et al., 2015).

2. Material and methods

2.1. Subjects

A total of 115 male OF1 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21 (4 animals were discarded during the training phase of SA). All mice (except those used as aggressive opponents) were housed in groups of five in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998) (n = 15 adult mice). All mice were housed under the following conditions: constant temperature, a reversed light schedule (lights off at 08:00 and on at 20:00), and food and water were freely available ad libitum, except during the behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees (University of Valencia).

2.2. Drugs

For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was dissolved in water.

2.3. Experimental design

A first set of mice were employed to corroborate that social stress produces neuroinflammation. Animals were sacrificed 3 h after the first exploration (Control group), the first and the fourth RSD and 3 weeks after the last exposure to RSD.

Our main objective was to confirm the increase of voluntary ethanol consumption in defeated animals and to evaluate the action of physical exercise on this social stress effect. Animals were exposed to RSD (RSD and RSD + Wheel groups) or exploration condition (EXP and EXP + Wheel). Furthermore, the EXP + Wheel and RSD + Wheel groups were exposed, individually and in a different cage from the usual mice home cage, to wheel activity three days a week from PND 40 until the end of the SA procedure. This groups trained in the wheel for 1 h before each RSD or exploration condition. Three weeks after the last exposure to RSD, the animals started the EtOH SA protocol for approximately 28 days. Brain samples were also obtained 24 h after the SA procedure.

2.4. Repeated social defeat

Animals in the stress/defeated groups were exposed to 4 episodes of RSD lasting 25 min each on PND 47, 50, 53 and 56. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the aggressive opponent (Covington and Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. The second phase of each RSD protocol was video-recorded and ethologically analyzed. Threat and attack behaviors were scored in aggressive opponent mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice. In the third phase, the wire mesh was put back for a further 10 min to allow social threats from the aggressive opponent. The non-stressed exploration groups underwent the same protocol, but without the presence of an aggressive opponent mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, after which the behavioral tests began (see Fig. 1).

In the corresponding groups, animals ran on the wheels immediately before each RSD or exploration (control group).

2.5. Apparatus and procedures

2.5.1. Oral ethanol self-administration

This procedure is based on the one employed by Navarrete et al. (2014). Oral ethanol SA was carried out in 7 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Packing software (Cibertec, SA, Spain) controlled stimuli and fluid delivery and recorded operant responses. The chambers were equipped with a chamber light, two nose-poke holes, one receptacle to deliver a liquid solution, one syringe pump, one stimulus light, and one buzzer and were placed inside noise isolation boxes. Active nose-poke delivered 36 μL of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6-s time-out period. The inactive nose-poke did not produce any

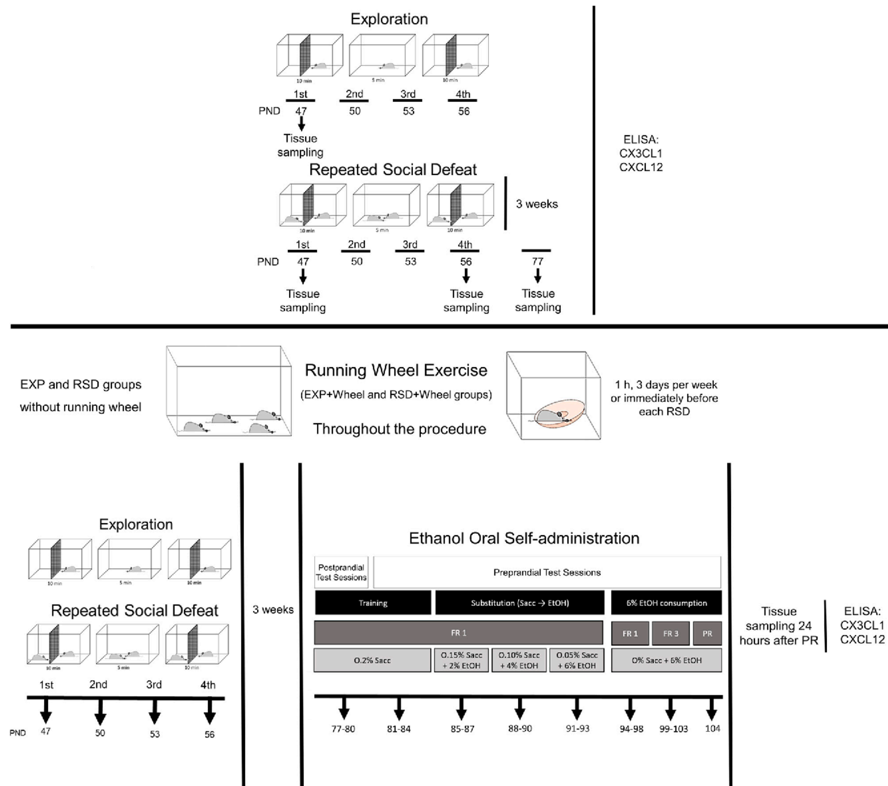

Fig. 1. Experimental design.

consequence.

To evaluate the consequences of RSD on the acquisition of oral EtOH SA, animals underwent an experiment carried out in three phases: training, saccharin fading and 6% EtOH consumption.

Training phase (8 days): Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withheld for 24 h, and food was provided 1 h prior to the 1 h session to increase the animals' motivation. During the subsequent 3 days, water was provided ad libitum, except during the 1-h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1-h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced in the mice weight loss of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 μL of 0.2 % (w/v) saccharin reinforcement.

Saccharin fading (9 days): The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac − 2% EtOH; 0.10% Sac − 4% EtOH; 0.05% Sac − 6% EtOH).

6% ethanol consumption (11 days): The aim of the last phase was to evaluate the number of responses on the active nose-poke, the 6% EtOH (w/v) intake and the motivation to drink. After each session, the alcohol that remains in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and EtOH consumption (μL) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, a fixed ratio 3 (FR3) (mice had to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement, according to the aforementioned scale (for example, if an animal activates the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40). All

the sessions lasted for 1 h, except the PR session, which lasted for 2 h.

2.5.2. Low-profile running wheel

The type of wheel used was the low-profile running wheel (Med Associates Inc.), which rotates on a central axis in a horizontal plane, allowing physical activity to be carried out through natural exercise as in spontaneous locomotion. These wheels have an ideal size (10.25 × 15.5 × 13.7) to be introduced into the home cages of rodents and are linked to a monitoring system (Hub) that runs on batteries and can register the activity through a set of programs (Wheel Manager Software). All mice were housed in groups of five in plastic cages throughout the experiment. However, mice in the exercise condition (EXP + Wheel and RSD + Wheel) were individually placed in a plastic cage different to their home cage with one low-profile running wheel. In our laboratory, we have eight low-profile running wheels. All animals in the exercise condition were distributed in batches of eight to run on the wheel for 1 h, three times a week (Monday, Wednesday, and Friday) or immediately before exposure to RSD.

2.6. Tissue sampling

Striatum samples were taken 3 h after the first and the fourth RSD. Likewise, another sample was taken three weeks later and a final sample was obtained after the end of the SA procedure.

To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed, the striatum dissected following the procedure described by Heffner et al. (1980) and kept in dry ice until storage at −80 °C. Before CX3CL1 and CXCL12 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 mL of cold lysis buffer (1% NP-40, 20 mM Tris−HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

2.7. Determination of CX3CL1 and CXCL12 levels

To determine the CX3CL1 and CXCL12 concentration on tissues, we used a Mouse CX3CL1 ELISA Kit obtained from Abcam (Ref: ab100683) and a Mouse CXCL12 Kit obtained from Abcam (Ref: ab100741) that were used following the manufacturer's instructions.

To determine the absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450 nm and final results were calculated using a standard curve following the manufacturer's instructions and expressed as ng/mg for CX3CL1 and as pg/mg for CXCL12 (tissues).

2.8. Statistical analysis

The data of the ethological analyses of opponent and intruder mice were analyzed by a two-way ANOVA with a one between-subjects variable—Exercise (with or without physical exercise)—and a one within variable—RSD encounter—with two levels: first and fourth RSD.

To analyze acquisition of EtOH SA, a three-way ANOVA was performed with a two between-subjects variable - Stress (EXP or RSD) and Exercise (with or without wheel access)—and a within-subjects variable—Days, with five levels of FR1 or FR3—followed by the Student's-Newman−Keuls test to compare the groups at different time points of the oral self-administration paradigm. A two-way ANOVA was employed to compare the effects of RSD on the number of active responses, breaking point values and ethanol consumption during PR with two between-subjects variable—Stress (EXP or RSD) and Wheel (with or without physical exercise).

Pearson's coefficient was calculated to determine possible relationships between the EtOH consumption variable (during FR1, FR3 or PR schedules) and ethological analyses of the behaviors exhibited by the intruder mice during RSD (first and fourth).

Data related to chemokine concentrations were analyzed by a one-way ANOVA. In the first set of animals, we analyzed the effects of RSD using an ANOVA with one between-subjects variable—Stress, with four levels (Control, first RSD, fourth RSD, 3 Weeks). For the second set of animals, after SA procedure, we used a two-way ANOVA, with two between-subjects variable—Stress (EXP or RSD) and Wheel (with or without physical exercise). The ANOVAs were followed by a Bonferroni's post-hoc test. The results are reported as mean ± S.E.M. All analyses were performed using SPSS v24.

Cohen's d effect sizes were calculated for all statistically different comparisons. Effect sizes were classified as small (d = 0.20–0.49), moderate (d = 0.50–0.79), and large ($d \geqslant 0.80$) (Cohen, 2013).

3. Results

3.1. VWR did not affect behaviors during RSD

The ANOVA revealed a significant effect of the variable Day for Defensive/Submissive [$F(1,18) = 50.932$; $p = 0.000$], for Attack [$F(1,18) = 16.357$; $p = 0.001$], and Threat [$F(1,18) = 5.872$; $p = 0.026$] behaviors (Table 1). All mice showed an increase of the time spent in these behaviors in the last RSD compared to the first ($p = 0.001$, $d = 2.244$; $p = 0.001$, $d = 1.290$; and $p = 0.026$, $d = 0.557$ respectively).

3.2. RSD increase CX3CL1 and CXCL12 levels in the striatum

The ANOVA indicated that the exposure to RSD induced a significant increase in CX3CL1 [$F(3,28) = 3.988$; $p = 0.017$] and CXCL12 [$F(3,28) = 5.304$; $p = 0.005$] protein levels in the Striatum (Fig. 2 a

Table 1
Ethological analyses of the RSD.

			Without exercise		With exercise (VWR)	
		Encounters	First	Fourth	First	Fourth
Intruder mice	Avoidance/flee	Time (s)	49 ± 6	49 ± 9	51 ± 7	45 ± 6
		Latency	8 ± 4	12 ± 10	5 ± 1	8 ± 3
	Defense/submission	Time (s)	51 ± 11	106 ± 10***	44 ± 7	105 ± 6***
		Latency	21 ± 13	5 ± 2	10 ± 2	5 ± 2
Opponent mice	Threat	Time (s)	14 ± 4	16 ± 3*	10 ± 7	13 ± 2*
		Latency	7 ± 3	6 ± 2	14 ± 11	20 ± 11
	Attack	Time (s)	54 ± 5	83 ± 11***	52 ± 5	84 ± 9***
		Latency	6 ± 3	2 ± 1	3 ± 1	3 ± 1

Results are presented as mean values ± SEM. *p < 0.05; ***p < 0.001 differences between first and fourth RSD.

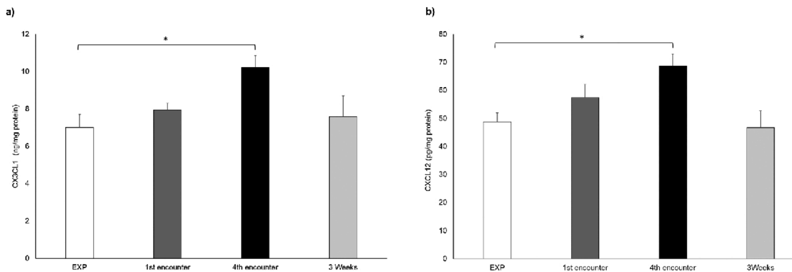

Fig. 2. RSD increase CX3CL1 and CXCL12 levels in the Striatum. (a) Concentration of CX3CL1 after exploration (EXP), first RSD, fourth RSD and 3 weeks after the last exposure to RSD. The columns represent the mean and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) of OF1 mice (n = 8 in all groups). (b) Concentration of CXCL12 after exploration (EXP), first RSD, fourth RSD and 3 weeks after the last exposure to RSD. The columns represent the mean and the vertical lines ± SEM of concentration levels of CXCL12 (pg/mg protein) of OF1 mice (n = 8 in all groups). *$p < 0.05$ with respect EXP group.

and b) after the fourth RSD compared to the control group ($p = 0.019$; $d = 1.831$ for CX3CL1; $p = 0.019$; $d = 1.998$ for CXCL12).

3.3. *VWR counteracts the increase in ethanol oral self-administration induced by RSD*

The analyses of the acquisition and substitution phases of the self-administration can be found on the supplementary data.

The ANOVA for the number of active responses during the FR1 schedule of EtOH SA revealed a significant effect of the interactions Days × Stress [$F(4,208) = 4.024$; $p = 0.032$] and Stress × Exercise [$F(1,52) = 4.959$; $p = 0.030$] (Fig. 2a). The post-hoc comparison showed that active responses were lower on day 1 compared to day 2 ($p = 0.05$; $d = 0.509$), 3 ($p = 0.001$; $d = 0.619$) and 5 ($p = 0.047$; $d = 0.356$) only in defeated animals. Defeated animals (RSD group) showed higher number of active responses than controls (EXP) ($p = 0.000$; $d = 1.31$), as well as the defeated mice exposed to wheels (RSD + Wheel) ($p = 0.000$; $d = 1.293$). With respect to EtOH consumption, the ANOVA revealed a significant effect of the interaction Stress × Exercise ([$F(1,52) = 10.124$; $p = 0.002$] (Fig. 2b). The post-hoc comparison showed that defeated animals (RSD) showed higher EtOH consumption rates than controls (EXP) ($p = 0.000$; $d = 1.566$) and the defeated mice exposed to wheels (RSD + Wheel) ($p = 0.000$; $d = 1.288$).

During the FR3 schedule, the ANOVA revealed a significant effect of the variable Days [$F(4,52) = 5.387$; $p = 0.000$] and variable Exercise [$F(1,52) = 4.959$; $p = 0.030$] for the number of active responses (Fig. 2a). The post-hoc comparison showed a lower number of active responses on day 1 with respect to days 2 ($p = 0.003$; $d = 0.461$), 3 ($p = 0.017$; $d = 0.417$) and 4 ($p = 0.023$; $d = 0.398$). A lower number of active responses was observed in animals that had had access to wheels (EXP + Wheel and RSD + Wheel) ($p = 0.030$; $d = 0.606$). With respect to EtOH consumption, the ANOVA revealed a significant effect on the interaction Days × Exercise [$F(4,208) = 3.546$; $p = 0.008$]. The post-hoc comparison showed that animals without access to a wheel (EXP and RSD) consumed significantly more EtOH with respect to animals with VWR on day 2 ($p = 0.000$; $d = 0.916$). In addition, EXP and RSD groups also showed a significant decrease in EtOH consumption during day 1 compared to days 2 ($p = 0.000$; $d = 0.928$), 3 ($p = 0.005$; $d = 0.570$) and 4 ($p = 0.011$; $d = 0.547$) (Fig. 3b).

During the progressive ratio, for breaking point values (Fig.3c) the ANOVA revealed a significant effect of the interaction Stress × Exercise [$F(1,52) = 4.379$; $p = 0.041$]. Post-hoc comparison showed that the breaking point values were higher in defeated animals with respect to the control group ($p = 0.019$; $d = 0.940$) and RSD + Wheel ($p = 0.010$; $d = 1.068$). The ANOVA for the numbers of rewards (Fig.3e) also revealed a significant effect of the variable Exercise [$F(1,52) = 8.281$] ($p = 0.006$), since the groups exposed to VWR showed a lower number of rewards ($p = 0.006$; $d = 0.773$). No effects were observed for EtOH consumption (Fig.3d).

3.4. *Passive coping during RSD correlated with stronger ethanol consumption during FR1 schedule*

Pearson's coefficient showed a positive correlation between the time spent in Avoidance/Flee behaviors during the 4th RSD and the average consumption of ethanol during FR1 schedule ($r = 0.699$; $p = 0.024$) in RSD group (Fig.4). Those mice that spent more time in avoidance/flee behaviors during RSD showed higher consumption rates of EtOH during the FR1 schedule.

3.5. *VWR reverses the increase in striatal levels of CX3CL1 and CXCL12 induced by RSD after EtOH SA*

The ANOVA revealed a significant effect of the interaction Stress × Exercise [$F(1,27) = 8.948$; $p = 0.006$] on CX3CL1 protein levels after oral SA of EtOH (Fig. 5a). The post-hoc comparison revealed that defeated animals (RSD) presented significantly higher levels of CX3CL1 than non-stressed animals (EXP) ($p = 0.009$; $d = 2.550$). In addition, defeated animals exposed to physical exercise (RSD + Wheel) showed significantly lower protein levels compared to defeated animals without access to a running wheel (RSD) ($p = 0.000$; $d = 4.803$).

Regarding CXCL12 protein levels, the ANOVA revealed a significant effect of the interaction Stress × Exercise [$F(1,27) = 4.849$; $p = 0.036$] after oral SA of EtOH (Fig. 5b). The post-hoc comparison revealed that the defeated group (RSD) showed significantly higher protein levels than the non-defeated group (EXP) ($p = 0.001$; $d = 2.213$). Higher levels of CXCL12 were obtained in the defeated group without access to the running wheel (RSD) compared to defeated group with VWR (RSD + Wheel) ($p = 0.002$; $d = 2.163$).

4. Discussion

The present study confirmed that social stress experienced during adulthood increases consumption and motivation for ethanol and that VWR reverted this effect. In addition, we corroborated that RSD produces a neuroinflammatory response by increasing protein levels of the chemokines CX3CL1 and CXCL12. VWR was also able to revert the neuroinflammatory response caused by stress and exposure to ethanol.

In a previous study from our laboratory, we observed that mice subjected to social stress during adolescence showed an increase in consumption and motivation for ethanol in the oral SA paradigm (Rodríguez-Arias et al., 2017). Those results agree with the results

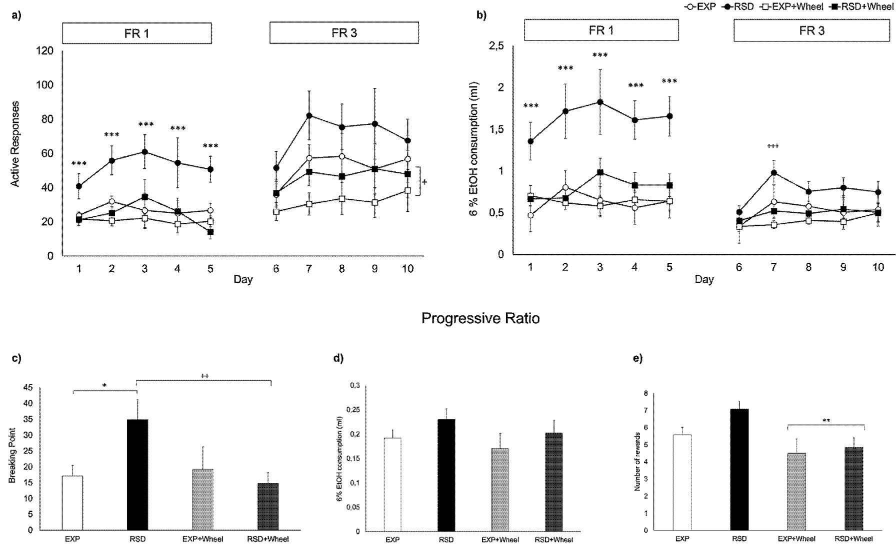

Fig. 3. Effects of running wheel on the increase in oral EtOH self-administration induced by RSD in OF1 mice. Animals were divided into the following four treatment groups: EXP group allowed to explore a new cage and without access to a running wheel (EXP, n = 14) or EXP group allowed to explore a new cage and with access to a running wheel (EXP + Wheel, n = 14); and RSD group exposed to RSD and without access to a running wheel (RSD, n = 15) or RSD group exposed to RSD and with access to a running wheel (RSD + Wheel, n = 13). The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% EtOH consumption and (e) the number of rewards obtained during PR. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ significant difference with respect to the control and RSD + Wheel groups; + $p < 0.05$, + + $p < 0.01$, + + + $p < 0.001$ significant difference between groups with running wheel access vs. groups without access.

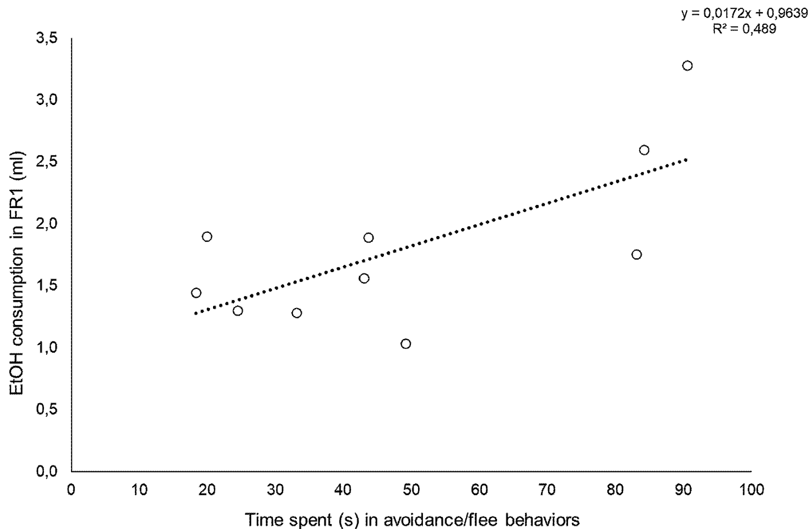

Fig. 4. Regression plot for the Pearson correlation between Avoidance/Flee behaviors (fourth RSD) and the average consumption of ethanol during FR1 schedule. The trend line represents the linear regression of data ($y = 0.017x+0,9639$; $r2 = 0.489$).

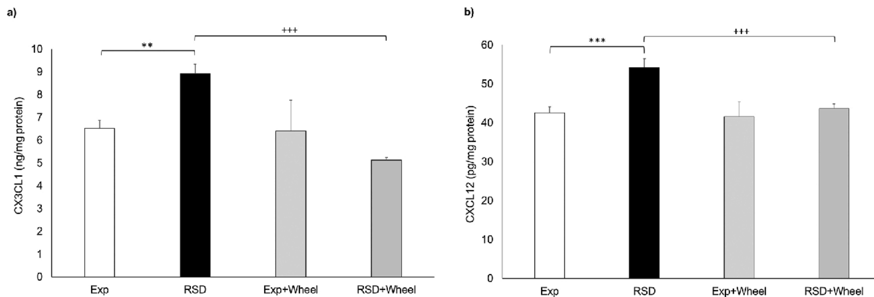

Fig. 5. VWR reverses the increase in striatal levels of CX3CL1 and CXCL12 induced by RSD after EtOH SA. (a) CX3CL1 protein levels and (b) CXCL12 protein levels after oral EtOH SA in the following four treatment groups: EXP group allowed to explore a new cage and without access to the running wheel (EXP, n = 8) or EXP group allowed to explore a new cage and with access to the running wheel (EXP + Wheel, n = 7); and RSD group exposed to RSD and without access to the running wheel (RSD, n = 8) or RSD group exposed to RSD and with access to the running wheel (RSD + Wheel, n = 8). The columns represent the mean and the vertical lines ± SEM of concentration levels of CX3CL1 (ng/mg protein) and CXCL12 (pg/mg protein) of OF1 mice. **$p < 0.01$, ***$p < 0.001$ significant difference with respect to the EXP group; $^{+++}p < 0.001$ significant difference with respect to the corresponding EXP or RSD groups.

obtained in this study in mice defeated during adulthood. The effect of social stress was more apparent during the FR1 phase, although increases were also observed during FR3. The greater the number of active responses needed, the less value the reward has, since there is a loss in the value of delayed rewards (Mazur, 1986; Lagorio and Winger, 2014). During the first day of FR3 a decrease in active responses is characteristically observed because the animals need to learn the new demands. Control non-stressed groups tend to decrease the interest in getting infusions, perhaps because their seeking behavior is not strong (Samson and Czachowski, 2003), although stressed animals work harder to obtain EtOH. Therefore, during the FR3 schedule, it is normal to observe a decrease in the number of infusions received and the total consumption of EtOH with respect to FR1, while defeated animals maintain a greater consumption. Equally, the PR schedule determines the breaking point or limit of active responses that the animal is willing to carry out to obtain EtOH. This progressive pattern of responses is usually indicative of seeking behavior related to motivation (Samson and Czachowski, 2003). We observed that defeated animals showed higher breaking points, although no more EtOH consumption was observed. Taking into account the progressive nature of PR schedules, a small number of substance intakes is usually observed due to the limited session time (in our case, 2 h) (Bickel et al., 1990). For this reason, it is difficult to observe differences in consumption. The PR schedule is complementary to the FR schedules, as it links the seeking, motivation, and maintenance behaviors of addictive behavior. In agreement with our results, other studies showed that rodents exposed to SD during adolescence (Marcolin et al., 2019) or adulthood (Deal et al., 2018; Lopez et al., 2016; Riga et al., 2018) showed an increase and escalation in voluntary consumption of ethanol.

Recent studies suggest that coping strategies are associated with resilience or vulnerability to stress (Wood et al., 2015; Chen et al., 2015; Finnell et al., 2017; Pearson-Leary et al., 2017), which seems to be related with neurochemical adaptations that specifically affect the function of the dopamine system and could therefore modify the rewarding efficacy of drugs of abuse (Brodnik et al., 2017). In the present study, we have observed that those animals that spent more time in avoidance and flee behaviors during the last SD presented higher EtOH intake. We have obtained similar results in a previous study (Ródenas-González et al., 2020), where we observed a positive correlation between flight and avoidance behaviors and the increase in the conditioned rewarding effects of cocaine in the CPP. These results confirm that active coping and adequate adaptation to stress reduces the increase in the rewarding effects of drugs of abuse induced by social stress. Other studies have also confirmed that mice showing active coping strategies show less anhedonia (Wood et al., 2015), less anxiety and greater social interaction (Duclot et al., 2011; Hollis et al., 2011; Kumar et al., 2014). In this work, we hypothesized that controlled physical activity could reduce the effects of RSD. VWR is a rodent model that mimics several aspects of human physical exercise training (Mul et al., 2018). There are no previous studies where VWR was used to intervene on ethanol consumption and motivation induced by social stress, but the effect of physical exercise on ethanol consumption has been studied in various paradigms of voluntary consumption of ethanol, such as TBC or the free-choice paradigm. In general, these studies show lower consumption rates of alcohol in male (Ehringer et al., 2009; Hammer et al., 2010) and female rodents (Piza-Palma et al., 2014) with access to the running wheel. However, a recent study pointed out that the removal of access to exercise appeared to enhance ethanol intake/preference (Lynch et al., 2019).

On the other hand, physical exercise has a well-documented beneficial effect on stress-related mental disorders. VWR counteracted the development of social avoidance and anhedonia after chronic SD stress (Mul et al., 2018; Zhang et al., 2019). Likewise, VWR attenuated the increased neuroendocrine response induced by social isolation stress (Watanasriyakul et al., 2019) and counteracted the behavioral impairments induced by uncontrollable stress (Greenwood et al., 2003, 2012, 2013; Tanner et al., 2019). The relationship between stress and exercise is bidirectional, as Parra-Montes de Oca et al. (2019) have recently reported that chronic stress decreases the metabolic response to voluntary exercise characterized by the loss of white adipose tissue depots.

Our study showed that exposure to VWR was capable of decreasing the long-lasting increase in ethanol intake induced by RSD. This counteracting action seems to be specific to the stress-induced effect, as no differences in ethanol intake were observed between the two non-stressed groups, meaning that exposure to VWR did not affect basal ethanol intake. In addition, the ethological analyses of RSD showed no differences in opponent or intruder mice behaviors depending on the exposure to VWR, meaning that previous exposure to exercise before each RSD did not affect social stress.

Inflammatory stimuli induced the release of inflammatory cytokines as well as chemokines that functioned as chemo-attractants, presenting homeostatic and/or inflammatory functions (Koper et al., 2018). SD have been linked to an increase of the neuroimmune response, including the activation of microglia (Wohleb et al., 2011, 2014; Lehmann et al., 2016; Rodríguez-Arias et al., 2018), the increase in BBB

permeability (Rodríguez-Arias et al., 2016), and the increase of IL-6 levels in plasma and the striatum (Ferrer-Pérez et al., 2018). We have now corroborated these results, showing that RSD also induced an increase in the chemokines CX3CL1 and CXCL12 after the fourth RSD.

There is no consensus whether CX3CL1 is an inflammatory or a pro-inflammatory chemokine (Rahman et al., 2011; Mecca et al., 2018). CX3CL1 signaling through its receptor Cx3cr1 which is only expressed in microglia, being critical for the microglia-neuron cross-talk (Jones et al., 2010; Lauro et al., 2015; Poniatowski et al., 2017). The stress-induced changes in CX3CL1 are not clear, as discrepant results have been described. Adult male rats exposed to chronic mild stress for 2 weeks not only showed an increase in CX3CL1 expression in the dorsal hippocampus, but also a decreased expression in the prefrontal cortex (Rossetti et al., 2016). Moreover, the same authors observed increases or decreases in CX3CL1 expression in the hippocampus after seven weeks of chronic mild stress. Although we have reported in the present study an increase of striatal CX3CL1 levels after the fourth RSD, we obtained in a recent report the opposite effect, following the same experimental procedure with a decrease in CX3CL1 levels in the striatum and no changes in the hippocampus (Montagud-Romero et al., 2020). The use of a different strain of mice (OF1 or C57BL/6NTac) could be responsible for these discrepant results. Since OF1 is a particularly territorial strain of mice, the loss of social encounters could have had a more intense stress effect leading to a higher neuroinflammation response. In addition, in the present study, we observed an increase in CX3CL1 levels in defeated mice after ethanol oral self-administration.

With respect to CXCL12, this chemokine is ubiquitously expressed and binding to two receptors, CXCR4 and ACKR3. The CXCL12/CXCR4/ACKR3 axis plays key roles in many physiological and pathological processes, including embryogenesis, wound healing processes, angiogenesis, homeostasis and it also participates in the progression of inflammation (McCandless et al., 2006; Niraula et al., 2018; García-Cuesta et al., 2019). Therefore, an increased expression of CXCL12 has been described in many inflammatory and autoimmune diseases (Wei et al., 2012; Rizzo et al., 2013), which suggests an inflammatory role. In agreement with this role, we observed that our RSD protocol induced a significant increase of this chemokine after the fourth defeat. However, Sawicki et al. (2015) did not observe changes in the CXCL12 gen-expression or a reduction of CXCL12 mRNA levels in enriched microglia/macrophages immediately after the last exposure to SD.

Many studies show that a prolonged consumption of ethanol produces increases in brain chemokine levels in rodents (Pascual et al., 2015; Somkuwar et al., 2016). In our study, both chemokines were significantly increased after oral ethanol SA in defeated animals in comparison with those non-stressed.

The increase in chemokines after EtOH oral SA, which was only found in the defeated group that had no access to VWR, suggests a sensitization of the neuroinflammatory response. Stressed mice exposed to ethanol presented higher levels of chemokines that are not observed in non-stressed animal or in those defeated but exposed to VWR. These differences could be due to the less amount of ethanol ingested by these mice, since it is well known that ethanol is per se a potent neuroinflammatory factor (Montesinos et al., 2016; Pascual et al., 2015). However, we have previously reported that defeated mice showed elevated levels of the pro-inflammatory cytokine IL-6 that were not observed in control mice after having been exposed to the same doses of cocaine (Ferrer-Pérez et al., 2019). Therefore, although we cannot prevent control mice to ingest less ethanol, we suggest that social stress sensitized the inflammatory system to further responses. Confirming these results, we have previously reported increases in these chemokines after cocaine administration in mice (Araos et al., 2015), In addition, we observed a decrease in CXCL12 chemokine in abstinent cocaine users without changes in CX3CL1 plasma levels, although these levels positively correlated with the cocaine symptom severity for cocaine abuse/dependence (Araos et al., 2015). Although we did not measure the acute effect of VWR after each RSD, we observed that after oral EtOH SA, VWR significantly decreased the striatal levels of chemokines (CX3CL1 and CXCL12), showing levels similar to those in the control group. In contrast with this effect, there are several reports showing an increased neuroinflammatory response in animals exposed to physical exercise, in some cases forced or maintained for 24 h a day (Svensson et al., 2016; Pinto et al., 2019). The literature suggests that moderate-intensity exercise may be optimal in decreasing neuroinflammatory markers (Henrique et al., 2018; Paolucci et al., 2018). In agreement with our results, some studies have shown the positive effects of controlled physical exercise on stress (Mul et al., 2018; Ignácio et al., 2019), addiction (Somkuwar et al., 2016) or Alzheimer's disease (He et al., 2017; Jensen et al., 2019; Małkiewicz et al., 2019). Physical exercise interacts with stress and neuroinflammation depending on the intensity. Several studies have observed that VWR reduces the levels of corticosterone and glucocorticoid receptors, attenuating the negative effects of chronic stress (Zheng et al., 2006; Ignácio et al., 2019; Lynch et al., 2019; Watanasriyakul et al., 2019). The inhibition of the excess production of corticosterone can attenuate the inflammatory response of stress (Niraula et al., 2018). Only few studies have examined the effect of exercise and chemokine levels. For example, long-term wheel performance decreases the western diet increased in the gene expression of CXCL10 and CCL2 (Carlin et al., 2016).

5. Conclusions

In conclusion, our results suggest that VWR is a beneficial environmental intervention that is capable of blocking the increased ethanol intake and the neuroinflammation induced by social stress. Our work highlights the complexity of the brain mechanisms involved in the inflammatory process in response to social stress. To sum up, VWR could be a promising preventive and therapeutic target to avoid and reduce the detrimental effects induced by social stress.

Data availability

The datasets generated for this study are available on request to the corresponding author.

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All the authors have made a substantial contribution for the conception, design, and drafting the article.

All the authors have approved the version to be submitted.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT authorship contribution statement

M.D. Reguilón: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. **C. Ferrer-Pérez:** Formal analysis, Investigation, Software, Validation, Writing - original draft. **R. Ballestín:** Investigation, Methodology. **J. Miñarro:** Conceptualization, Funding acquisition, Project administration,

Resources, Supervision, Writing - review & editing. **M. Rodríguez-Arias:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

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Annex 3: Study 3. Ethanol intake in male mice exposed to social defeat: Environmental enrichment potentiates resilience

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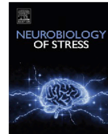

Ethanol intake in male mice exposed to social defeat: Environmental enrichment potentiates resilience

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ABSTRACT

Large preclinical evidence shows that exposure to social defeat (SD) increases vulnerability to drug abuse, increasing the consumption of ethanol. However, not all subjects are equally affected by the changes induced by stress. Previous reports have evidenced that the resilient phenotype to depressive-like behaviors after SD is associated with the resistant phenotype to cocaine-increased rewarding effects and the smaller neuro-inflammatory response. The aim of the present study was to further clarify whether the resilient profile to depressive-like behavior also predicts a protection against the increase in ethanol intake induced by SD. The neuroinflammatory profile was studied after the end of the oral ethanol self-administration (SA) procedure, measuring levels of the pro-inflammatory cytokine IL-6 and the chemokine CX3CL1 or fractalkine in the striatum and prefrontal cortex. Previous studies have shown that environmental enrichment (EE) is an effective mechanism to dimish the detrimental effects of social stress. In a second study, we aimed to evaluate if EE housing before exposure to SD could potentiate resilience. Our results showed that mice with a phenotype susceptible to SD-induced depressive-like behaviors showed increased ethanol consumption and increased neuroinflammatory signaling. In contrast, despite the lack of effect on depressive-like behaviors, defeated mice previously housed under EE conditions did not show an increase in ethanol SA or an increase in immune response. To sum up, the resilient phenotype to SD develops at different levels, such as depressive-like behaviors, ethanol consumption and the neuroinflammatory response. Our results also point to the protective role of EE in potentiating resilience to SD effects.

1. Introduction

We are continuously exposed to different types of stress throughout our life, and stress produced by social interaction is the most common type of stress in human beings (Montagud-Romero et al., 2018). Numerous studies have shown that exposure to social stress is associated with an increase in drug use, such as cocaine (Ferrer-Pérez et al., 2018; Reguilón et al., 2017; Rodríguez-Arias et al., 2016, 2017), MDMA (García-Pardo et al., 2015) or alcohol (Beutel et al., 2018; Hwa et al., 2016; Montagud-Romero et al., 2021; Newman et al., 2018a; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Regarding alcohol, the studies to date show that both exposure to stress and the way to cope with it should be considered as predictors of alcohol consumption in humans (see review by Newman et al., 2018b). People who consume alcohol in a negative context, for example to reduce anxiety or stress, are more likely to develop a long-term problematic use and develop a chronic alcohol consumption with the corresponding negative consequences that characterize long-term alcohol abuse (e.g. negative social, physical and mental consequences; Newman et al., 2018a; Sinha, 2001). Moreover, exposure to social stress can further increase the likelihood of developing uncontrolled alcohol use or relapse (Adinoff et al., 2017).

The social defeat (SD) model is the most widely used model to study the effects of social stress (Hammels et al., 2015). SD consists of an agonistic encounter between conspecifics of the same species (Miczek et al., 2004), imitating the subordination status of human relationships (Selten et al., 2013). Exposure to SD stress induces profound physiological changes and endocrine responses, yielding a significant increase in corticosterone levels (Montagud-Romero et al., 2015; Rodríguez-Arias et al., 2017). In addition, it produces modifications in numerous

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Abbreviations

AUD	alcohol use disorder	IL:	infralimbic cortex
BDNF	brain-derived neurotrophic factor	IL-6	interleukin 6
CPP	conditioned place preference	NAc	nucleus accumbens
CRF	corticotrophin-releasing factor	NLRP3	Nod-like receptor pyrin containing 3
CX3CL1	C-X_3-C motif ligand 1 (fractalkine)	PFC	prefrontal cortex
EE	environmental enrichment	PND	postnatal day
ELISA	enzyme-linked immunosorbent assay	PR	progressive ratio
FR1	fixed ratio 1	PrL:	prelimbic cortex
FR3	fixed ratio 3	SA	self-administration
HPA	hypothalamic-pituitary-adrenal	SD	social defeat
		SWR	social withdrawal ratio
		TLR-4	Toll-like receptor 4

neurotransmitter systems such as the serotonergic, dopaminergic or the GABAergic systems (Montagud-Romero et al., 2018).

Using this procedure, we have previously shown that exposure to four SD episodes either during adolescence or adulthood induced a long-lasting increase of ethanol self-administration (SA) during adulthood (Montagud-Romero et al., 2021; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Adolescent or adult mice were exposed to four SD episodes on alternating days and, three weeks after the last encounter, we measured oral ethanol SA. Defeated mice showed a delayed increase in ethanol consumption, made more active responses and showed increased motivation for alcohol in the progressive ratio (PR). Our results and other similar results obtained with voluntary ethanol drinking (Hwa et al., 2016; Norman et al., 2015) or indicating increased sensitivity to ethanol –induced conditioned place preference (CPP; Macedo et al., 2018)– confirmed that social stress increases vulnerability to the rewarding effects of alcohol.

Besides increasing alcohol intake, animals exposed to SD also exhibit increased anxiety and depressive-like behaviors, such as social avoidance (Blanco-Gandía et al., 2019; Ferrer-Pérez et al., 2019; Patel et al., 2019; Spijker et al., 2020). In the last decade, numerous studies have observed that the behavioral and psychological reactions to SD are not equal. Some animals are more susceptible and develop unhealthy responses, such as increased drug intake, anxiety or depressive-like behaviors. However, other subjects show resilience to stress and present a more adjusted psychological functioning (Brockhurst et al., 2015; Charney, 2004; Dantzer et al., 2018; Krishnan et al., 2007; Nasca et al., 2019).

Numerous studies have shown that passive, rather than active, coping strategies during SD are linked to stress-induced maladaptive behaviors (Ballestín et al., 2021; Hawley et al., 2010; Russo et al., 2012; Wood and Bhatnagar, 2015). Animals that display passive coping mechanisms seem to be susceptible to physiological effects and psychopathology (Hawley et al., 2010; Russo et al., 2012; Wood and Bhatnagar, 2015). However, the stress response does not only involve the coping strategies during stress, but also its physiological processes (Murrough and Russo, 2019). The link between individual differences and the immune system response to stress is now a critical field of research (Ballestín et al., 2021; Hodes et al., 2014; Westfall et al., 2021; Wood et al., 2015). As a general result, these pre-clinical studies report lower immune system responses to depressive-like behaviors induced by social stress in resilient mice, compared to susceptible animals that developed anhedonia or social withdrawal behavior. Moreover, social stressors can modify the brain's reward system function due to the close association between the brain systems that regulate stress and the systems responsible for responses to drugs of abuse (Rodríguez-Arias et al., 2013). In contrast to the numerous reports that focus on predicting the individual response to depression-like stress consequences, only a recent study focused on the resilience and susceptibility to stress-induced enhancement of the cocaine response. We showed that resilient mice to depressive-like behaviors are also resilient to the increased cocaine reward induced by SD and exhibit a less intense neuroinflammatory response (Ballestín et al., 2021).

Although the increase in psychostimulant effects induced by SD has been thoroughly studied in the literature, there are fewer studies regarding the increased ethanol intake, and to our knowledge, only one study has focused on the resilient response to SD. Riga et al. (2020) suggest that resilience to depressive-like behaviors could protect from the development of alcohol use disorder (AUD)-like phenotypes. In their study, rats classified as depression-prone were more vulnerable to alcohol, emulating patterns of alcohol dependence as those seen in individuals with an alcohol use disorder. In this study, animals were exposed to repeated SD, and subsequently isolated for several weeks. Their depression profile was evaluated during isolation, weeks after the last defeat. In addition to social avoidance, cognitive performance was also used to further classify animals into resilient or susceptible to depressive-like behaviors. Although the authors claimed that depression-prone animals showed a more intense pattern of alcohol consumption, their increase in alcohol intake during SA acquisition was not significantly higher. However, they observed a greater response to alcohol reward during the fixed ratio 3 (FR3) and PR schedule. In addition to high motivation toward alcohol, these depression-prone rats showed a tendency toward extinction resistance and relapse facilitation.

The present study was designed to further clarify if the resilient profile to depressive-like behavior also predicts a protection against the increase in ethanol intake induced by SD. The neuroinflammatory profile of resilient and susceptible mice were also studied after the end of the oral SA procedure, measuring levels of the pro-inflammatory cytokine interleukin 6 (IL-6) and the chemokine C-X3-C motif ligand 1 (CX3CL1) or fractalkine in the striatum and the prefrontal cortex (PFC). Both neuroinflammatory markers were reported to be differentially affected by social stress experiences in resilient or susceptible animals (Ballestín et al., 2021; Reguilón et al., 2020, 2021). To further characterize the potentiation of the resilience response, a second experiment took place to evaluate the effect of environmental enrichment (EE) exposure during adolescence, prior to the SD stress. The EE model selected for this work can be considered a basic and modest EE model, which based on the results obtained previously (Giménez-Gómez et al., 2021), we hypothesize is sufficient to stimulate resilience and block the increase in the reinforcing effects of ethanol and the neuroinflammatory response induced by SD.

2. Methodology

2.1. Animals

A total number of 87 adult male C57BL/6 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. Experimental mice were housed in groups of four in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N = 20) and

were individually housed in plastic cages (21 × 32 × 20 cm) for at least one month prior to initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (red light from 8:00 to 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (number 2017-VSC-PEA-00224, on December 11th, 2017).

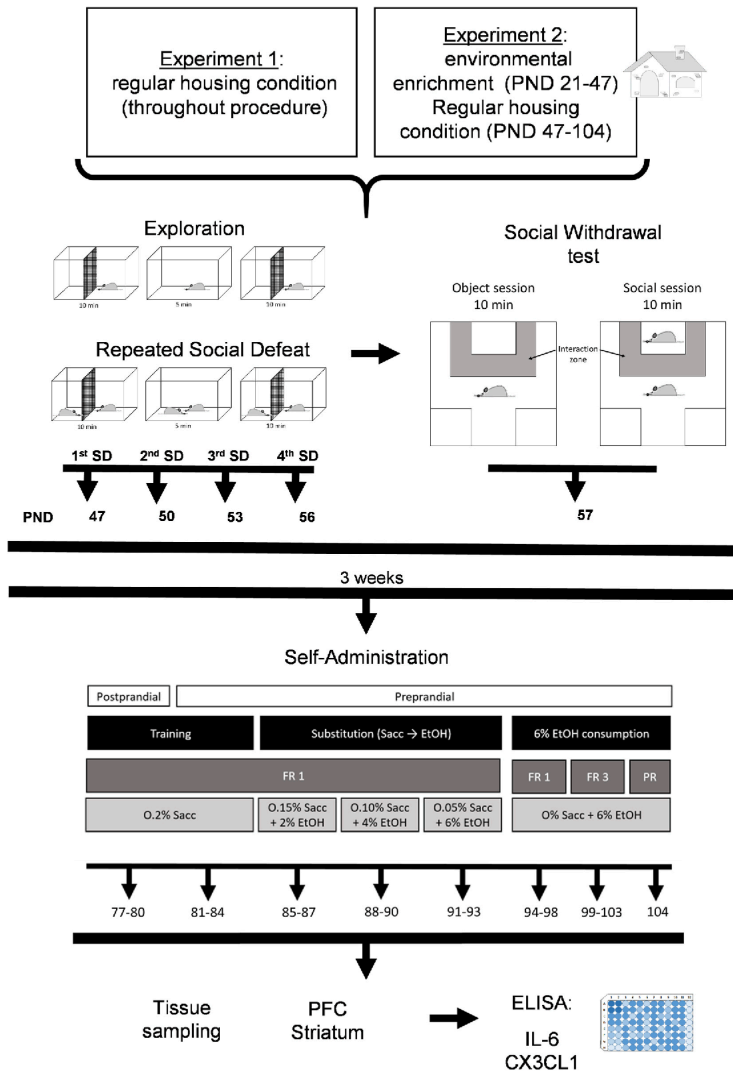

Fig. 1. Experimental design.

2.2. Drugs

For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was dissolved in water.

During the SA training phase, a 0.2% (w/v) saccharin solution in water was used. During the SA substitution phases, a mixture of 0.15% saccharin concentration dissolved in water and 2% ethanol was used for the first subphase; in the second subphase, a mixture of 0.10% saccharin solution in water and 4% ethanol was used; and, in the third subphase, a mixture of 0.05% saccharin solution in water and 6% ethanol was used.

2.3. Experimental design

The study consisted of two experiments. The experimental design between the two experiments differs only in the housing condition of the animals. In the first experiment, all the animals were housed in regular condition throughout the study. In the second experiment, all mice were housed in a consistent EE in big cages (59 x 38 × 20 cm) with PVC items such as plastic houses and tubes from PND 21 to 47. The day before the beginning of SD, mice housed in EE were moved to standard housing conditions until the end of the SA procedure, i.e., the animals were only exposed to EE from the onset of adolescence until early adulthood or late adolescence.

All mice were exposed to the SD procedure or exploration from PND 47 to 56 (i.e., during early adulthood or late adolescence). 24 h after the last SD episode, animals performed the Test for Social Interaction to evaluate depressive-like behaviors and were characterized as resilient or susceptible depending on their social withdrawal ratio (SWR). Subsequently, three weeks after the last defeat, the animals initiated the ethanol SA protocol for approximately 28 days. At the end of this test, all the animals were sacrificed to obtain the PFC and striatum for further analysis of the cytokine and chemokine levels.

The experimental design is depicted in Fig. 1.

2.4. Procedure and apparatus

2.4.1. Housing conditions

Male mice in the regular housing condition were housed in groups of four in transparent plastic cages (27 × 27 × 14 cm) with no more enrichment than standard bedding (wood flakes 1–3.35 mm), nesting material (paper strands) and two wooden gnaw sticks (5 x 1 × 1 cm) per cage. Male mice in EE conditions were housed in groups of four in plastic cages (59 x 38 × 20 cm) with standard bedding and nesting material, two wooden gnaw sticks plus additional PVC tunnel (13 × 5.5 cm) and a plastic mouse house (12.5 x 10.5 × 11 cm; Ferrer-Pérez, 2019; Giménez-Gómez et al., 2021).

2.4.2. Procedure of social defeat (SD)

Animals in the stress/defeated groups were exposed to 4 episodes of SD during adulthood, each lasting 25 min and consisting of three phases. The initial phase began by introducing the “intruder” (the experimental animal) into the home cage of the “resident” (the aggressive opponent) for 10 min (Tornatzky and Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington and Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two animals over a 5-min period. Finally, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a “resident” mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodríguez-Arias et al., 1998).

2.4.3. Social withdrawal ratio (SWR)

The SWR used was based on the social approach-avoidance test previously described by Berton et al. (2006). The test took place 24 h after the last SD during dark cycle and in a different environment of the confrontation sessions. First, animals were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Animals were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Although it can be argued that the probe mouse used in the social interaction test resembles the aggressor, and that this could foster social aversion, this is unlikely, since previous experiments demonstrate similar amounts of social investigation, irrespective of the strain used (i.e., C57BL/6; Berton et al., 2006). Before each session, the arena was cleaned with 5% alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Locomotion and arena occupancy during object and social sessions were determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage. Corners were defined as two squares of similar areas on the opposite wall of the arena.

2.4.4. Apparatus and procedures: Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 36 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% ethanol consumption.

2.4.4.1. Training phase (8 days). Two days before the initiation of the experiment, access to the standard diet was restricted to 1h per day. Before the first training session, water was withdrawn for 24h, and food allotment was provided 1h prior to the session to increase the motivation for active nose-poking. During the subsequent three days, water was provided ad libitum, except during the 1h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1h after the end of each daily session and water was available ad libitum to avoid ethanol consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding

weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 μl of 0.2% (w/v) saccharin reinforcement.

2.4.4.2. Saccharin substitution (9 days). The saccharin concentration was gradually decreased as the ethanol concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% ethanol; 0.10% Sac −4% ethanol; 0.05% Sac −6% ethanol).

2.4.4.3. 6% ethanol consumption (11 days). The aim of the last phase was to evaluate the number of active nose-poke responses, the 6% ethanol (w/v) intake and the motivation to drink. This phase began 38 days after the last SD. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and ethanol consumption (μl) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, FR3 (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a PR session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale. For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40. All the sessions lasted 1 h, except the PR session, which lasted 2 h (Navarrete et al., 2012, 2014).

2.4.5. Immunoassay analysis (ELISA)

Samples from the striatum and the PFC were obtained 24 h after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Paxinos and Franklin, 2001), which were then kept in dry ice until storage at −80 °C. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). We determined CX3CL1 and IL-6 concentration in the striatum and PFC. All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the final results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

2.5. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SWR. SWR is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups. To study the relationship between the percentages of susceptible mice in non-enriched and enriched mice, the chi-square (χ^2) test was used to evaluate the categorical variables Stress and Housing.

To analyze acquisition of ethanol SA, a two-way ANOVA was performed with one between-subjects variable –Stress with three levels (Control, Resilient and Susceptible; or EE-Control, EE-SD-R and EE-SD-S)– and a within-subjects variable –Days, with five levels of FR1 or FR3–. The effects of SD and treatment on breaking point values and ethanol consumption during PR was analyzed by a two-way ANOVA, with one between-subjects variable –Stress.

The data of the CX3CL1 and IL-6 levels were analyzed using a one-way ANOVA with one between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible; or EE-Control, EE-SD-R and EE-SD-S).

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics v.26. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

In order to evaluate the differences induced by housing conditions (standard housing and environmental enrichment), we additionally performed a statistical analysis with the variable Housing for the 6 groups. For ethanol SA, we performed a two-way ANOVA with two between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible) and Housing, with two levels (SH and EE)– and for FR1 and FR3, a within-subjects variable –Days, with five levels–. The effects of SD and housing on the breaking point values and the ethanol consumption during PR, as well as the results of striatum protein levels of IL-6 and CX3CL1, were analyzed by a two-way ANOVA, with two between-subjects variables –Stress and Housing.

3. Results

3.1. Resilience to SD under regular housing conditions

3.1.1. Classification between susceptible and resilient mice according to their social withdrawal ratio

Following the SWR calculation criteria, the control group ($n = 12$) showed a mean SWR higher than 1.

In the defeated group of animals ($n = 30$), 53.3% of the mice showed a SWR under 1, which classifies them as susceptible mice ($n = 14$), and the remaining 46.6% of the mice showed a SWR equal to or higher than 1, which classifies them as resilient mice ($n = 16$).

3.1.2. Susceptible mice showed higher ethanol intake than resilient animals

No differences were found between the animals during training or substitution phases, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Days [$F(1,39) = 17.697$; $p < 0.001$] and Stress [$F(2,39 = 4.854$; $p < 0.01$] (Fig. 2a). The post-hoc comparison showed that mice performed fewer active responses on days 1 and 2 compared to the last day ($p < 0.001$ in all cases). Moreover, susceptible mice performed fewer active responses

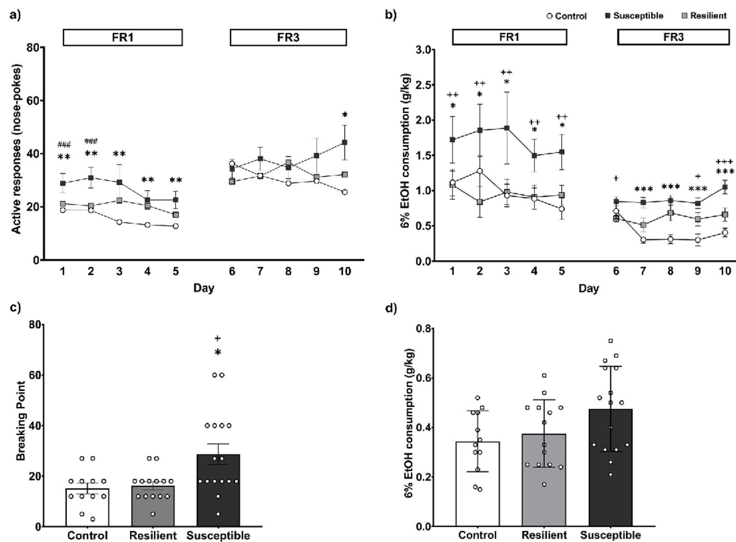

Fig. 2. Resilient mice showed lower ethanol intake than susceptible animals. Mice were divided into Control (n 12); Resilient (n 14) and Susceptible (n 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% ethanol consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% ethanol consumption during PR. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ significant difference with respect to controls; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ significant difference with respect to resilient mice. ###$p < 0.001$ significant difference with respect to day 5.

than the control group ($p < 0.01$).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Stress [$F(2,35) = 4.650$; $p = 0.01$) (Fig. 2b). The post-hoc comparison showed that the susceptible group consumed ethanol at higher rates than the control (p's < 0.05) and resilient groups ($p < 0.01$).

During the FR3 schedule, the ANOVA revealed a significant effect of the interaction Days × Stress [$F(4,184) = 4.940$; $p = 0.001$) for the number of active responses (Fig. 2a). Susceptible mice showed a higher number of active responses than controls on day 10. Moreover, control animals showed a lower number of active responses on day 10 compared to day 6 ($p < 0.05$). However, susceptible mice increased the number of active responses on days 6 and 8 compared to day 10 ($p < 0.05$ and $p < 0.01$, respectively).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Days [$F(4,156) = 5.216$; $p < 0.001$], Stress [$F(2,39) = 3.949$; $p < 0.001$] and the interaction Days × Stress [$F(8,156) = 3.691$; $p < 0.001$] (Fig. 2b). Susceptible mice consumed significantly more ethanol than controls on days 7–10 ($p < 0.001$ in all cases) and than resilient mice on days 6 ($p < 0.05$), 9 ($p < 0.05$) and 10 ($p < 0.001$). Moreover, control animals consumed more ethanol on day 6 compared to the rest of the days ($p < 0.001$ in all cases). Susceptible mice also consumed more ethanol on day 10 compared to the previous days ($p < 0.01$ with respect to days 6, 7 and 9; $p < 0,05$ with respect to day 8).

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the variable Stress [$F(2,39) = 6.418$; $p < 0.004$] (Fig. 2c). The post-hoc comparison showed that the breaking point values were higher in susceptible mice with respect to control and resilient animals ($p < 0.01$ in both cases). The ANOVA for ethanol consumption during PR did not reveal a significant effect of the variable Stress (Fig. 2d).

3.1.3. Susceptible mice showed altered levels of cytokine IL-6 and chemokine CX3CL1

The ANOVA for the striatal IL-6 levels showed an effect of the variable Stress [$F(2,37) = 9.957$; $p < 0.001$] (see Fig. 3a). Susceptible mice displayed higher IL-6 levels than the controls ($p < 0.001$), and resilient animals ($p < 0.01$). The ANOVA for IL-6 levels in PFC showed an effect of the variable Stress [$F(2,37) = 4.283$; $p < 0.021$] (see Fig. 3c). Susceptible mice displayed higher IL-6 levels than control animals ($p < 0.05$) without differences with resilient animals.

The ANOVA of striatal CX3CL1 levels revealed a significant effect of the variable Stress [$F(2,37) = 4.807$; $p < 0.014$] (see Fig. 3b). Striatal CX3CL1 levels were lower in susceptible animals in comparison with controls ($p < 0.01$). The ANOVA of CX3CL1 levels in PFC also revealed a significant effect of the variable Stress [$F(2,37) = 13.037$; $p < 0.007$] (see Fig. 3d). CX3CL1 levels in PFC were lower among all defeated animals (either resilient or susceptible) in comparison with controls ($p < 0.05$ for resilient and $p < 0.001$ for susceptible).

3.2. Environmental enrichment effects on resilience to SD

3.2.1. Environmental enrichment did not increase the percentage of resilient mice depending on the social withdrawal ratio

Following the SWR calculation criteria, the control group exposed to EE ($n = 14$) showed a mean SWR higher than 1.

In the defeated group of animals with EE ($n = 31$), 51.6% of mice showed a SWR under 1, which classifies them as susceptible mice ($n = 16$), and the remaining 48.4% of mice showed a SWR equal to or higher than 1, which classifies them as resilient mice ($n = 15$).

The comparison between the percentage of susceptible mice in the

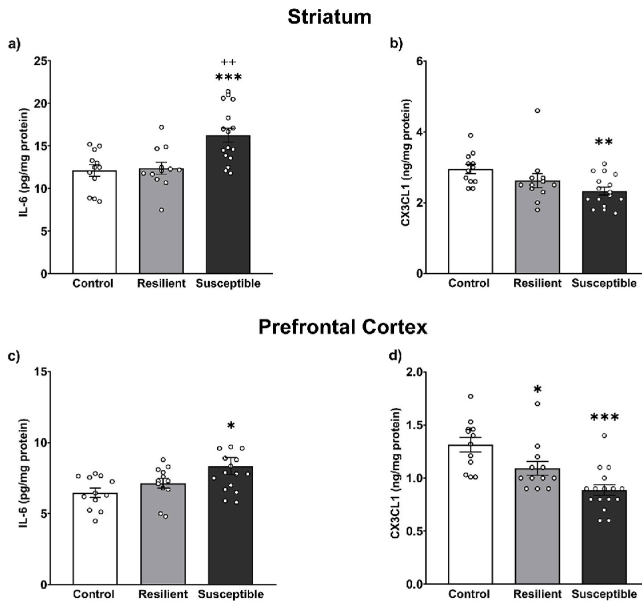

Fig. 3. Effect of repeated SD on IL-6 and CX3CL1 levels in the striatum and PFC. Bars represent mean pro-inflammatory cytokine IL-6 (in pg/mg) and chemokine CX3CL1 levels (in ng/mg) in the striatum (a and b) and PFC (c and d) and vertical lines ± SEM. Mice were divided into Control (n 12); Resilient (n 14) and Susceptible (n 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. *p < 0.05, **p < 0.01, ***p < 0.001 significant difference with respect to the control; ++ p < 0.01 significant difference with respect to resilient mice.

non-enriched experiment (53.3%) and the percentage of susceptible mice in the enriched experiment (51.6%) showed no statistical difference ($\chi^2(1) = 0.018$; $p = 0.893$; Table 1).

3.2.2. *Adolescent exposure to environmental enrichment reduces ethanol intake in susceptible animals*

No differences were found between the animals during the training and substitution phases, showing that EE and SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Stress (Fig. 4a), with susceptible mice making more active responses than control animals ($p < 0.05$). During the FR3 schedule, the ANOVA revealed a significant effect of the variable Days [$F(4,168) = 4.215$; $p < 0.01$] (Fig. 4a). Mice performed less active responses on the 10th day compared to the 7th ($p < 0.01$), 8th ($p < 0.01$), and 9th ($p < 0.05$) days.

With respect to ethanol consumption, the ANOVA of the g/kg of ethanol intake during the FR1 and FR3 schedule of ethanol SA did not reveal any significant effect of the variable Days or Stress (Fig. 4b), meaning that defeated mice, either resilient or susceptible, did not consume more ethanol than non-stressed control animals.

During the PR, the ANOVA for the breaking point values of ethanol SA and for ethanol consumption did not reveal any significant effect of the variable Stress (Fig. 4c and d).

Table 1
Housing condition and classification in the Social Interaction Test of defeated mice.

		Housing		Total
		Non-EE	EE	
Stress				
Susceptible mice	n	16	16	32
	%	53.3	51.6	52.5
Resilient mice	n	14	15	29
	%	46.7	48.4	47.5
Total	n	30	31	61
	%	100	100	100

3.2.3. *Environmental enrichment diminishes the neuroinflammatory response in susceptible mice*

The ANOVA of striatal IL-6 (Fig. 5a) and CX3CL1 (Fig. 5b) levels did not reveal any significant effect of the variable Stress.

3.2.4. *Environmental enrichment vs standard housing condition*

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Housing [$F(1,78) = 4.451$; $p < 0.001$]. The post-hoc comparison showed that the enriched mice performed higher active responses than the non-enriched mice ($p < 0.05$). With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Housing [$F(1,75) = 6.050$; $p < 0.05$). The post-hoc comparison showed that the standard-housed mice consumed ethanol at higher rates than the enriched mice ($p < 0.05$).

During the FR3 schedule, the ANOVA revealed a significant effect of the interaction Days × Stress × Housing [$F(8,300) = 2.717$; $p < 0.01$) for the ethanol consumption. The standard housed group consumed significantly more ethanol than the control enriched group on day 6 ($p < 0.001$). The resilient standard-housed group consumed significantly more ethanol than the resilient enriched group on days 6, 7 (p's < 0.05), 8 ($p < 0.001$), 9 ($p < 0.01$) and 10 ($p < 0.001$). Moreover, the susceptible standard-housed group consumed significantly more ethanol than the susceptible enriched group on days 6, 7, 8, 9 and 10 (p's < 0.001).

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the interaction Stress × Housing [F

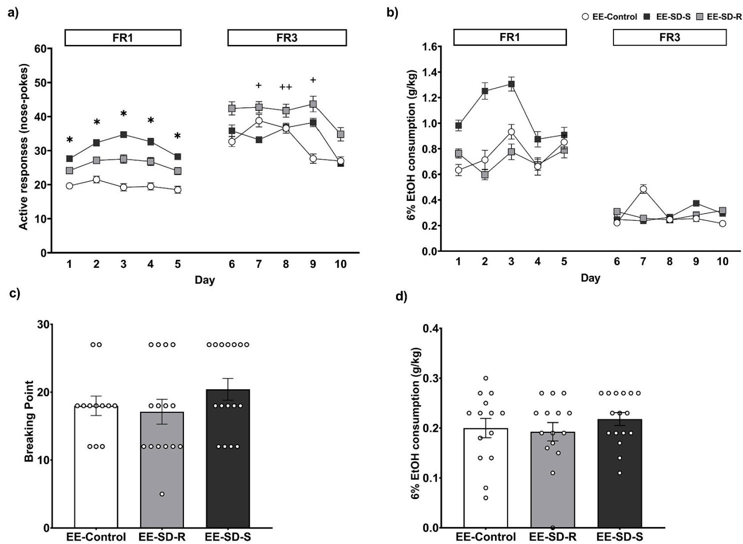

Fig. 4. Environmental enrichment reduces ethanol intake in susceptible animals. Mice were divided into EE-Control (n = 14); EE-SD-R (n = 15) and EE-SD-S (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the volume of 6% ethanol consumption during FR1 and FR3. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, (d) the volume of 6% ethanol consumption during PR. *p < 0.05, significant difference with respect to controls; + p < 0.05, ++ p < 0.01 significant difference with respect to the 10th day.

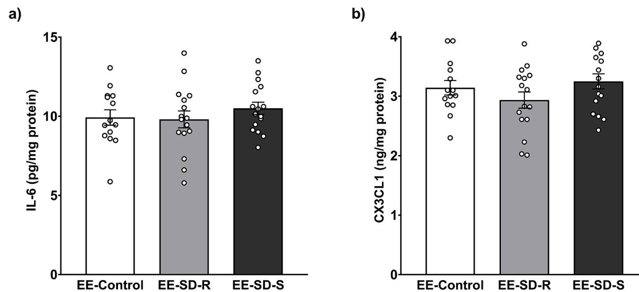

Fig. 5. Environmental enrichment reduces levels of the pro-inflammatory cytokine IL-6 and chemokine CX3CL1 in susceptible mice. Bars represent the mean of the striatal IL-6 (a) levels (in ng/kg) and CX3CL1 (b) levels (in pg/mg) and the vertical lines ± SEM. Mice were divided into EE-Control (n = 14); EE-SD-R (n = 15) and EE-SD-S (n = 16). Defeated mice were characterized as resilient or susceptible depending on their SWR.

(2,79) = 3.052; $p = 0.05$]. The post-hoc comparison showed that the breaking point values were higher in susceptible standard-housed mice with respect to the susceptible enriched animals ($p < 0.001$). The ANOVA for ethanol consumption during PR revealed a significant effect of the variable Housing [$F(1,81) = 65.766$; $p < 0.001$]. The post-hoc comparison showed that enriched mice consumed significantly less ethanol than standard-housed mice ($p < 0.001$).

The ANOVA for the striatal IL-6 levels showed an effect of the interaction Stress × Housing [$F(2,80) = 5.278$; $p < 0.01$]. The ANOVA revealed that the control standard-housed group displayed higher IL-6 levels than the control enriched group ($p < 0.05$). The susceptible standard-housed mice showed higher IL-6 levels than the susceptible enriched mice ($p < 0.001$). In the same line, the resilient standard-housed mice showed higher IL-6 levels than the resilient enriched mice ($p < 0.01$).

4. Discussion

It is well known that the behavioral and neurobiological effects of social stress are not equally manifested in all individuals. Most of the studies have focused on the particular response to depressive-like behaviors and neuroinflammation (Nasca et al., 2019; Pfau and Russo, 2015). However, few studies have evaluated the resilience/susceptibility response to drug abuse after social stress. We have recently reported that resilient mice to depressive-like behaviors also show a resilient response to the increased cocaine reward induced by SD, which is accompanied by a lower neuroinflammatory response (Ballestín et al., 2021). In the present study, we further confirm that mice presenting a phenotype resistant to depressive-like behaviors are also unaffected by the increased ethanol intake induced by SD. Defeated resilient mice did not show any increase in ethanol intake, conversely to those susceptible, which also showed increased motivation for ethanol in the PR. These resilient mice developed minor neuroinflammatory responses with lower levels of IL-6 and higher levels of CX3CL1 in the PFC and the striatum than their susceptible counterparts. To further unravel the mechanisms of the resilient response, we evaluated the protective role of EE housing during adolescence before exposure to SD. Although EE during adolescence did not increase the percentage of resilient mice to depressive-like behaviors evaluated through the SWR, neither resilient nor susceptible mice increased their oral ethanol SA consumption. Moreover, none of the defeated mice exposed to EE developed any increase in neuroinflammatory markers. One limitation of the study is that the effects produced by SD in female rodents have not been evaluated in this work. Due to the sex differences observed in female mice with respect ethanol intake, it is necessary to perform suitable models of social stress for female rodents to address this issue in the future.

4.1. Resilience to the increase in ethanol intake induced by social defeat

The typical measure to classify animals as resilient or susceptible to SD effects is the SWR. Investigation of a social target is a natural behavior in healthy rodents; therefore, social avoidance is considered a depressive-like behavior. In the social interaction test, performed 24 h after the last SD, susceptible mice are stressed animals that display social avoidance.

Numerous studies have shown that SD induces changes in the reward system, affecting drug intake. With regard to ethanol, exposure to SD increases the conditioned rewarding effects of ethanol using the CPP paradigm (Macedo et al., 2018). Studies of voluntary ethanol consumption have observed increased and escalating consumption of ethanol, as well as an increased motivation to drink alcohol, in defeated animals using the oral SA paradigm (Barchiesi et al., 2021; Montagud-Romero et al., 2021; Norman et al., 2015; Reguilón et al., 2020, 2021; Rodríguez-Arias et al., 2016). Using other paradigms such as the two-bottle choice, an increase in SD-induced escalation of alcohol intake has also been observed (Croft et al., 2005; Deal et al., 2018; Hwa et al., 2016; Newman et al., 2018a).

We have previously reported that animals classified as susceptible to SD-induced depressive-like behaviors were also susceptible to the increased rewarding effects of a subthreshold dose of cocaine three weeks after the last SD (Ballestín et al., 2021). In the present study, we confirmed that the resilient or susceptible phenotype to depressive-like behaviors induced by SD also correlates to the ethanol intake phenotype. In contrast to resilient mice, those classified as susceptible depending on the SWR test showed a higher increase in ethanol consumption and motivation to obtain a reward. There is only one other study evaluating this relation. Riga et al. (2020) evaluated the effects of a long-term SD-induced depressive phenotype, subsequently followed by a period of social isolation, on alcohol-seeking and drinking behaviors in male rats. This study presents significant differences with regards to ours, such as the use of two different social stressors, the SD and social isolation. The authors performed five SD exposures for five consecutive days and the intruder mice were immediately housed in isolation for the rest of the study. On the other hand, mice in our study were subjected to four intermittent sessions of SD and were socially housed throughout the study. It is known that the intensity, duration and number of exposures influence the intensity of subsequent behavioral symptoms and long-term effects on substance abuse (Shimamoto, 2018). Another important difference lies in the criterion to characterize the animals as resilient or susceptible to SD-induced depressive-like behaviors, which was based on social approach-avoidance and the object place recognition tests during the isolation period. Although these authors did not observe any increase in ethanol intake during the SA acquisition and FR1, susceptible rats exhibited a significant increase in alcohol responsiveness during FR3 and a higher motivation to drink alcohol during the PR schedule compared to the control group. Susceptible rats also showed a higher number of extinction sessions and a higher relapse than non-stressed animals. Although no differences in ethanol consumption during FR1 were observed in the work of Riga et al. (2020), susceptible rats performed a higher number of active responses. One possible explanation for the lack of difference in ethanol intake could be the higher ethanol concentrations used (12%). We can hypothesize that SD may change the sensitivity to ethanol preference, with stressed animals being more sensitive to a low ethanol concentration, such as the 6% used in our study. In addition, social isolation is known to induce profound behavioral and neurobiological alterations (Mumtaz et al., 2018). Besides inducing anxiety and depressive-like behaviors in rodents (Amiri et al., 2015), several studies pointed that isolation induces an increase in ethanol consumption in mice and rats (Advani et al., 2007; Evans et al., 2020; Juárez and Vázquez-Cortés, 2003; Lopez et al., 2011; Sanna et al., 2011).

4.2. Susceptible mice showed increased levels of IL-6 and CX3CL1

Numerous studies have shown that ethanol activates the innate immune system by stimulating Toll-like receptor 4 (TLR4) signaling in glial cells, triggering the release of inflammatory mediators and causing neuroinflammation (Alfonso-Loeches et al., 2010; Ibáñez et al., 2019; Montesinos et al., 2016; Pascual et al., 2015). The induction of astrogliosis and microgliosis increases the release of cytokines (IL-1β, IL-6, IL-17, TNF-α) and the production of chemokines (MCP-1, MIP-1α, CX3CL1), causing brain damage in various brain structures such as the PFC, striatum, hippocampus and cerebellum (Alfonso-Loeches et al., 2010; Bachtell et al., 2015; Drew et al., 2015; Fernandez-Lizarbe et al., 2009; Guerri and Pascual, 2019; Pascual et al., 2011, 2018; Vetreno and Crews, 2015). In addition to the neuroinflammatory response, alcohol exposure diminishes cell proliferation, migration, growth and differentiation, even causing cell death (Alfonso-Loeches and Guerri, 2011).

The brain areas analyzed in this study play a crucial role in the addictive cycle. On the one hand, the PFC controls subcortical regions to drive motivated behavior (Koya et al., 2009; West et al., 2014). The PFC consists of subregions that appear to mediate different aspects of the addiction cycle. For example, the prelimbic area (PrL) or dorsal area in rats projects preferentially to the Nucleus Accumbens (NAc) core, and the infralimbic (IL) or ventral area projects preferentially to the NAc shell (Heidbreder and Groenewegen, 2003; Ongür and Price, 2000). PrL appears to play a critical role in cue-elicited drug seeking (Lasseter et al., 2010), and IL appears to be primarily involved in inhibiting drug seeking (Peters et al., 2008). On the other hand, the striatum also consists of subnuclei involved in different stages of the addictive cycle. The ventral striatum (NAc) is associated with incentive salience pathways and salience attribution, i.e., it has been associated with the reinforcing actions of drugs of abuse (Koob, 2015; Koob and Volkow, 2010). While the dorsal striatum is related to habit formation (stimulus-response habit learning), and therefore, is key in the development of habitual compulsive drug use (Koob, 2015; Koob and Volkow, 2010).

Exposure to social stress promotes an increase in the neuroimmune response. Numerous preclinical studies show that SD is accompanied by

the activation of neuroinflammatory events, including microglial activation and increased cytokine production (Calcia et al., 2016; Ferrer-Pérez et al., 2018; Finnell and Wood 2016; Montagud-Romero et al., 2021; Rodríguez-Arias et al., 2017, 2018; Wohleb et al., 2011, 2012, 2014). In addition, SD promotes the deterioration of the blood-brain barrier (BBB), and a decrease in the expression of the tight binding protein claudin-5, laminin and collagen-IV has been observed in the hippocampus and NAc (Menard et al., 2017; Rodríguez-Arias et al., 2017). Using the same procedure to induce SD as in the present study, we have observed that exposure to SD can induce a long-lasting increase in the concentration of pro-inflammatory cytokines such as IL-6 in the PFC, striatum and hippocampus (Ballestín et al., 2021; Ferrer-Pérez et al., 2018) and a significant upregulation of the protein pro-inflammatory markers NFkBp-p65, IL-1β, IL-17 A and COX-2 in the striatum of male mice (Montagud-Romero et al., 2021). An increase of chemokines such as CX3CL1 and CXCL12 in the striatum and PFC of defeated mice has also been observed (Reguilón et al., 2020, 2021), although a decrease in CX3CL1 protein levels in the hippocampus and striatum (Ballestín et al., 2021; Montagud-Romero et al., 2020) has also been described using another strain of mice. Both pro-inflammatory and anti-inflammatory functions for CX3CL1of have been described (Mattison et al., 2013; Sheridan and Murphy, 2013; Zujovic et al., 2000), since the CX3CL1-CX3CR1 signaling has a neuroprotective function and maintains communication between neurons and microglia (Sheridan and Murphy, 2013). CX3CL1 seems to have anti-inflammatory effects mainly (Lyons et al., 2009; Zujovic et al., 2000), and an efficient CX3CL1 signaling between neurons and microglia appears to be critical for the protection of social stress-induced depressive-like behaviors. For example, CX3CR1 KO mice showed an exaggerated HPA axis response to social stress (Winkler et al., 2017).

Moreover, individual differences in the neuroinflammatory mechanisms observed after SD stress have been described. When characterized as susceptible to the depressive-like behaviors induced by SD, these animals showed increased levels of cytokines IL-6, MCP-1 or IL-1β (Hodes et al., 2014; Stewart et al., 2015; Wood et al., 2015), with an increase of anti-inflammatory cytokines IL-4 and IL-10 in resilient rodents (Hodes et al., 2014; Stewart et al., 2015). A recent study observed that exposure to chronic unpredictable mild stress triggered a significant increase in Nod-like receptor pyrin containing 3 (NLRP3) expression only in susceptible mice, but not in resilient mice. These changes were accompanied by altered levels of IL-1β expression (Yang et al., 2021). Increases in both the NLRP3 and IL-1β expressions are associated with the development of depressive-like behaviors (Felger and Lotrich, 2013; Raison and Miller, 2013). Moreover, chronic SD caused a significant decrease in cAMP levels in the NAc neurons of susceptible mice (Zhang et al., 2020), promoting BBB permeability. These results indicate that stress resilience may be associated with reduced pro-inflammatory signaling, and suggest that therapeutic treatment on these pathways could promote stress resilience (Yang et al., 2021). However, only a recent study from our laboratory evaluated if this neuroinflammatory response is also observed in mice susceptible to the increased cocaine reward induced by SD. We observed that these mice exhibited elevated neuroinflammatory levels of the pro-inflammatory cytokine IL-6 and a decrease in the chemokine CX3CL1 in the striatum and hippocampus after being exposed to SD (Ballestín et al., 2021). Moreover, striatal and hippocampal IL-6 levels continued to be elevated more than 5 weeks after the last SD in susceptible mice. In the present study, we have corroborated and extended these results. After oral ethanol SA, susceptible mice showed increased IL-6 levels in the striatum and PFC. In addition, a decreased CX3CL1 was equally observed in both structures after SA in susceptible mice, although resilient animals also showed a decreased CX3CL1 in the PFC. To our knowledge, this is the first study showing that animals susceptible to the increased rewarding effects of ethanol induced by SD showed a long-lasting increase in the neuroinflammatory response.

4.3. EE promotes resilience to the effects of SD on alcohol intake and the neuroinflammatory response

In the second study, mice were housed in an enriched environment during adolescence (PND21), but housed under standard housing conditions from the first SD (PND47) until the end of the experiment. In other words, our objective was to determine the existence of a protective effect of EE on depressive-like behavior and the long-term vulnerability to the rewarding effects of ethanol and the neuroimmune response induced by SD. Our results confirmed the protective effect of EE in ethanol intake and in the neuroinflammatory response induced by SD.

EE has been typically associated with an improved well-being, increased cognitive function and a potentiation of stress resilience, and different models of EE have been used in order to reduce vulnerability to the detrimental effects of SD. However, the results observed in the literature are discrepant. In mice housed in EE and then subjected to 7 days of daily SD, an increase in aggressiveness and anxiety has been described, probably derived from a change in social stability (McQuaid et al., 2013a, 2013b). In these studies, EE not only did not decrease the neuroinflammatory response, but it even increased the corticotropin-releasing factor (CRF) levels in the PFC in both stressed and control mice.

Nevertheless, other studies found that EE is active in diminishing the neurobiological and behavioral effects induced by social stress, indicating that housing conditions may modulate the impact of external stressors. EE reduces acute and chronic stress-induced anxiety-like behaviors and cognitive impairments (Bahi, 2017; Cordner and Tamashiro, 2016; Dandi et al., 2018; Marianno et al., 2017). In addition, animals under EE housing show minor corticosterone increases and neuronal activation after a stressful experience (Branchi et al., 2013; Mesa-Gresa et al., 2016; Reichmann et al., 2013).

In contrast with the previously presented studies, we applied an EE prior to the exposure to SD to determine whether this housing condition during adolescence could potentiate the resilient response to depressive-like behavior and increase ethanol intake induced by this kind of stress. Our results showed that exposure to EE prior to SD does not influence SD-induced depressive-like behavior evaluated by SWR. In this way, we observed the same percentage of resilient and susceptible animals according to this score among those housed in EE when comparing with those from the first experiment housed under standard conditions. However, stressed resilient and susceptible mice housed in EE during adolescence did not show any long-lasting increase in ethanol intake or motivation to get the drug after SD. A recent study of Seo et al. (2021) observed that early exposure to EE is capable of blocking depressive-like behaviors induced by chronic unpredictable stress when animals are housed under standard conditions. In addition, previous housing under EE prevented epigenetic changes induced by this stressor. Although, as in our study, mice were exposed to EE during adolescence, there are important methodological differences between both studies. In Seo's study, mice were housed in EE for a longer period, but more importantly, they used a different type of stressor named chronic unpredictable stress. Finally, we employed mice of the OF1 strain, which are particularly affected by SD due to their high territoriality. All these differences could be responsible for the discrepant results in EE in preventing depression-like behaviors. Despite the lack of a standardized EE model, we consider the model employed in this investigation to be promising. Exposure to EE during adolescence has favored and enhanced adaptive behaviors in the face of subsequent exposure to social stress.

The role of EE in reducing ethanol intake has been widely demonstrated. For example, Rodríguez-Ortega et al. (2018) proved that housing adult mice in EE reduces ethanol binge intake, and likewise, social and environmental enrichment reduced ethanol preference (Holgate et al., 2017). However, there are few studies evaluating the therapeutic potential of EE on the reinforcing and motivational effects of ethanol induced by SD. Bahi (2017) observed that the increased anxiety-like behavior, the increase in ethanol intake and the appearance of

ethanol-induced CPP were buffered by exposure to EE conditions after the stress experience. In a more recent study, EE also proved to counteract social stress effects favoring the extinction of memories associated with ethanol and reducing reinstatement of drug seeking (Bahi and Dreyer, 2020).

EE also modulated the neuroinflammatory response induced by SD in the striatum. We did not observe any changes in IL-6 or CX3CL1 levels in the striatum in any of the groups evaluated. Differently with the first set of animals, susceptible mice housed in EE did not show any increase in IL-6 levels in the striatum, as observed in susceptible animals housed under standard housing conditions. Moreover, CX3CL1 levels also did not decrease in these mice, as it did in susceptible mice in the first study. As we did not observe any differences in the striatum of mice housed under EE conditions, the area most closely related to rewarding behavior, we did not analyze PFC. These results suggest that exposure to EE before SD reduces the impact of long-term social stress on the neuroinflammatory response, acting as a protective factor. There are no similar studies evaluating the neuroinflammatory response of social stress and ethanol consumption applying EE models, but our results are in line with studies evaluating the effect of EE on the neuroinflammatory response of social stress. Attenuations of the increase in IL-6 and IL-β1 in the prefrontal mRNA expression induced by moderate social stress have been observed in animals under EE conditions (McQuaid et al., 2018).

Among the beneficial effects of EE that could account for the protective effect on the increased ethanol intake and the neuroinflammatory response, we should highlight an increase in neurogenesis with an elevated expression of brain-derived neurotrophic factor (BDNF; Novkovic et al., 2015; Schloesser et al., 2010) and an enhanced synaptic and transcriptomic capacity (Hüttenrauch et al., 2016; Zhang et al., 2018). Exposure to EE during adolescence could also change the dynamics of social interaction, sensory processing and the mechanisms underlying baseline stress, with a decrease in CRHR1 genes and an increase in hippocampal CRHR2 observed in male rats housed in EE conditions (Kentner et al., 2018). Among other factors, facilitation in problem-solving ability and oxytocin immunoreactive responsiveness induced by EE in male rats must also be taken into consideration (Neal et al., 2018).

5. Conclusions

To sum up, our results corroborate that SD produces depressive-like behaviors, increased reinforcing and motivational effects of ethanol and induced greater neuroinflammatory response in susceptible mice, contrary to what occurs in resilient animals. The susceptible phenotype for depressive-like behaviors predicts the increased reinforcing and motivational effects of voluntary ethanol consumption and a larger neuroinflammatory response almost 2 months after the last SD exposure. In addition, we demonstrate that EE promotes the development of adaptive responses to social stress, indicating the importance of exposure to complex environments during adolescence.

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CRediT authorship contribution statement

Marina D. Reguilón: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Carmen Ferrer-Pérez:** Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Carmen Manzanedo:** Methodology, Project administration, Resources, Supervision, Writing – review & editing. **José Miñarro:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Marta Rodríguez-Arias:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no possible conflict of interest in the carrying out and reporting of this research.

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Annex 4: Study 6. Resilience to social defeat stress in adolescent male mice

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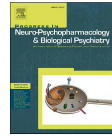

Resilience to social defeat stress in adolescent male mice

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ABSTRACT

Adverse social experiences during adolescence are associated with the appearance of mental illness in adulthood. Social defeat (SD) is an ethologically valid murine model to study the consequences of social stress. In adolescent mice, SD induces depressive-like behaviors, increased anxiety and potentiates the reinforcing effects of cocaine and alcohol. However, not all mice exposed to SD will be susceptible to these effects. Adult mice resilient to the effects of SD show a consistent phenotype being resilient to depressive-like behaviors and to the increase in cocaine and alcohol consumption. The aim of the present study was to characterize the resilient phenotype to depressive-like behaviors and increase cocaine and ethanol rewarding effects of mice socially defeated during adolescence. To that end, adolescent mice were exposed to repeated SD, and 24 h after the last encounter, they underwent a social interaction test (SIT) in order to evaluate depressive-like behaviors. Cocaine-induced reward conditioning and ethanol intake was evaluated in two different sets of mice 3 weeks after the last SD using cocaine-induced conditioned place preference (CPP) and oral ethanol self-administration (SA). The neuroinflammation response was measured at the end of the experimental procedure by measuring striatal and cortical levels of IL-6 and CX3CL1. The results confirmed that a comparable percentage of adolescent mice develop resilience to depressive-like behaviors to that observed in adult mice. However, increased anxiety was more severe in resilient mice. Likewise, an increased preference for an ineffective dose of cocaine and an increased ethanol consumption was observed in resilient mice compared to controls. The increase in IL-6 and CX3CL1 was mainly observed in the striatum of susceptible mice compared to that of control mice. Our results confirm that, contrary to prior assumptions in adults, responses to SD stress are more complex and singular in adolescents, and caution should be taken for the correct interpretation and translation of those phenotypes.

1. Introduction

Adolescence is a critical period of development characterized, among other behaviors, by an increase in the time spent with peers, a change in the quality of social interaction and frequent appearance of feelings of rejection (Platt et al., 2013; Somerville et al., 2010). Adverse social experiences during adolescence have been strongly associated with the appearance of mental illness in adulthood. Many subjects who have suffered from abuse or have been abandoned by their parents during developmental periods are diagnosed in adulthood with a mental illness such as depression, anxiety or drug addiction (Ho and King, 2021). A recent meta-analysis reported that adverse childhood or adolescent experiences are highly associated with anxiety and depression, costing upwards of billions of dollars annually (Bellis et al., 2019). But the strongest association is found with problematic drug use and interpersonal and self-directed violence (Hughes et al., 2017). Specifically, clinical studies indicate that stressful adolescent experiences increase the risk for substance abuse (Tharp-Taylor et al., 2009; Topper et al., 2011). Due to the close relationship between the brain systems involved in the response to drugs and stress, environmental stressors can produce long-term changes in the brain reinforcement system, inducing the individual to use drugs (Rodríguez-Arias et al., 2013).

Animal models enable the study of the mechanisms through which environmental and psychosocial stressors induce later neuropsychiatric disorders. Social defeat (SD) is considered the most representative

Abbreviations: BP, breaking point; CPP, conditioned place preference; DA, dopamine; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus maze; FR1, fixed ratio 1; FR3, fixed ratio 3; HPA, hypothalamic- pituitary- adrenal; PFC, prefrontal cortex; PND, postnatal day; Post-C, postconditioning; PR, progressive ratio; Pre-C, preconditioning; SA, self-administration; SD, social defeat; SIT, social interaction test; VTA, ventral tegmental area.

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animal model to study the consequences of social stress (Hammels et al., 2015). SD is an ethologically valid murine model that induces long-term physiological and behavioral changes similar to those seen in depression and anxiety and can mimic the individual differences in the stress response observed in humans (Wang et al., 2021).

SD in adult mice potentiates the reinforcing effects of different drugs, producing an increased intake of cocaine and other psychostimulants on the self-administration (SA) and conditioning of place preference (CPP) paradigms (Ballestín et al., 2021; Covington 3rd et al., 2008; Ferrer-Pérez et al., 2019; Giménez-Gómez et al., 2021; Quadros and Miczek, 2009; Montagud-Romero et al., 2016, 2020; Montagud-Romero et al., 2021; Reguilón et al., 2017; Rodríguez-Arias et al., 2017). A significant increase in alcohol consumption after exposure to SD has also been reported (Montagud-Romero et al., 2021; Reguilón et al., 2020, 2021a; Reguilón et al., 2021b; Rodríguez-Arias et al., 2016). However, the effects of SD during adolescence on subsequent drug abuse or mental health have not been widely investigated.

Neural and behavioral development of rodents is thought to mirror stages of human development (Adriani and Laviola, 2004; Burke and Miczek, 2014). Like adolescent humans, adolescent rodents are highly social, to a greater extent than adult rodents (Do Couto et al., 2009; Yates et al., 2013). Defeated adolescent rats or mice show reduced social behavior, depressive-like behaviors, or increased anxiety similar to what is observed in defeated rodents in adulthood (Huang et al., 2013; Iñiguez et al., 2014; Shimizu et al., 2020). Similar to adults, increases in cocaine SA (Burke and Miczek, 2015), amphetamine-, cocaine-, and alcohol-induced conditioned reinforcement (Burke et al., 2011; Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017), and oral ethanol SA (Burke and Miczek, 2015; Rodríguez-Arias et al., 2016: Thompson et al., 2020) are observed in socially defeated adolescent rodents.

Nevertheless, not all subjects exposed to stress will develop depressive, anxiety or addictive behaviors. But as in humans, a subset of mice exposed to SD will be susceptible to these effects, developing important disorders such as social inhibition, anhedonia or depressive-like behaviors (Krishnan et al., 2007). However, some rodents will be resilient to these consequences, being able to adaptively cope with stress (Cathomas et al., 2019). We have recently reported that mice resilient to the effects of SD during adulthood show a consistent phenotype; that is, these mice are resilient to the depressive-like behaviors produced by SD, and are also resilient to the reinforcing effects of cocaine and alcohol (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). The response to SD seems to be much more complex in adolescent mice, and this also appears to be the case in the development of an adaptive response to stress. To date, only two studies have evaluated their resilience profile. Both studies reported that only a small proportion of the defeated adolescent mice (between 20 and 30%) were totally susceptible or totally resilient to certain effects of SD. However, these studies did not address the increased susceptibility to drug abuse (Alves-dos-Santos et al., 2020; Vassilev et al., 2021).

The aim of the present study was to characterize the resilient phenotype to depressive-like behaviors and the increased rewarding effects of cocaine and ethanol SA in socially defeated mice during adolescence. To that end, adolescent mice were exposed to repeated SD and, 24 h after the last encounter, underwent a social interaction test (SIT) in order to evaluate depressive-like behavior. Cocaine-induced reward conditioning and ethanol intake was evaluated in two different sets of mice 3 weeks after the last social defeat using cocaine-induced CPP and oral ethanol SA.

Recent studies suggest that the neuroinflammatory response may play an important role in the development of mental illness (Liu et al., 2020a; Soria et al., 2018), as the immune system also regulates the hypothalamic-pituitary-adrenal axis (HPA), thereby modulating the response to a stressful situation (Haroon et al., 2012). It is well known that social stress induces an activation of the immune system with short- and long-term increases in the levels of cytokines and chemokines (Ferle et al., 2020; Ferrer-Pérez et al., 2018; Jiang et al., 2020; Nozaki et al., 2020; Montagud-Romero et al., 2020; Reguilón et al., 2020; Reguilón et al., 2021a). These results have also been confirmed with SD in adolescent mice, which also showed an impairment of integrity in the blood-brain barrier and activation of the microglia (Rodríguez-Arias et al., 2017; Rodríguez-Arias et al., 2018; Zhu et al., 2019). We observed that this neuroinflammatory response is absent in socially defeated mice during adulthood that showed a resilient phenotype to depressive-like behaviors and increased cocaine or ethanol intake (Ballestín et al., 2021; Reguilón et al., 2021b). Therefore, we will also characterize the neuroinflammatory response in defeated adolescent mice after cocaine or ethanol exposure, measuring the IL-6 and CX3CL1 level in the striatum and the prefrontal cortex (PFC).

In summary, we aim to characterize the behavioral response to the conditioned rewarding effects of cocaine and ethanol intake, as well as the neuroinflammatory response in mice with a resilient phenotype to the depressive-like effects induced by social defeat during adolescence.

2. Material and methods

2.1. Subjects

A total number of 77 adolescent male C57BL/6 J mice (Charles River, France) were used in this study. The experimental mice (PND 21) were housed in groups of five in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents ($N = 20$) and were individually housed in plastic cages (21 × 32 × 20 cm) for at least a month prior to the initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). All mice were housed in controlled laboratory conditions: constant temperature and humidity, and a reversed light schedule (lights off at 08:00 and on at 20:00). Food and water were available ad libitum to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (2019/VSC/PEA/0059 y 2019-VSC-PEA-122).

2.2. Drugs

For CPP, a dose of 1.5 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Spain) was employed and injected intraperitoneally (i.p.). This dose of cocaine was selected based on previous CPP studies showing that doses below 3 mg/kg are sub-threshold (Arenas et al., 2014; Montagud-Romero et al., 2017; Vidal-Infer et al., 2012). Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drug. For the oral SA procedure, absolute ethanol (Merck, Madrid, Spain) was dissolved in water using a *w/v* percentage, i.e. a 6% (*w/v*) ethanol solution equivalent to a 7.6% (*v/v*) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water.

2.3. Experimental groups and experimental design

In this study, two different sets of mice were employed, all of which were exposed to the SD procedure or exploration from PND 27 to 36. 24 h after the last SD episode, on PND 37, all the mice performed the elevated plus maze (EPM) and the SIT to evaluate depressive-like behaviors. Subsequently, the first set of mice underwent the CPP procedure with 1.5 mg/kg of cocaine on PND 57, after 3 weeks of being undisturbed in their home cages. Mice were characterized as resilient or susceptible depending on their ratios in the SIT. Brain samples were taken at the end of the procedure (PND 65).

Likewise, after performing the SDs, EPM and SIT, the second set of mice initiated the 6% oral ethanol SA protocol on PND 57, 3 weeks after the last defeat, lasting approximately 28 days. During this paradigm, the mice proceeded through the phases of training (7 days), substitution of saccharin for ethanol (10 days), the FR1 (5 days), FR3 (5 days) and PR

(1 day) schedules. At the end of this test, all the mice were sacrificed to obtain the brain samples for further analysis (PND 85).

The experimental design is depicted in Fig. 1.

2.4. *Apparatus and procedures*

2.4.1. *Procedure of social defeat (SD)*

Mice in the stress/defeated groups were exposed to 4 episodes of SD during adolescence, each lasting 25 min and consisting of three phases. The initial phase began by introducing the "intruder" (the experimental mice) into the home cage of the "resident" (the aggressive opponent) for 10 min (Tornatzky and Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington 3rd and Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two mice over a 5-min period. Finally, the wire mesh was put back in the cage to separate the two mice once again for a further 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in the cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define a mouse as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). All agonistic encounters of each SD protocol were videotaped to confirm SD of the intruder mice and to ethologically analyze the threat and attack behaviors (duration and latency) of the resident mice. These behaviors were scored in resident mice and avoidance/flee and defensive/submissive behaviors were evaluated in intruder mice.

2.4.2. *Elevated plus maze (EPM)*

The EPM test was carried out essentially following the procedure described by Daza-Losada et al. (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, mice were habituated to the experimental room for 1 h prior to testing. At the beginning of each trial, the experimental mice were placed on the central platform facing an open arm and were allowed to explore for 5 min. The behavior displayed by the mice during the test was recorded by an automated tracking system (EthoVision XT 11, Noldus) that tracks the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms were measured to characterize the anxiolytic effects of the SD (Ferrer-Pérez et al., 2018; Rodríguez-Arias et al., 2016).

2.4.3. *Social interaction test (SIT)*

The social withdrawal ratio used was based on the social approach-avoidance test previously described by Berton et al. (2006). The test took place 24 h after the last SD during the dark cycle and in a different environment from the confrontation sessions. First, mice were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each mouse was placed in the center of a square arena (white Plexiglas open field, 30 cm each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 11, 50 fps; camera placed above the arena). Mice were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first session (object session), an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Although it can be argued

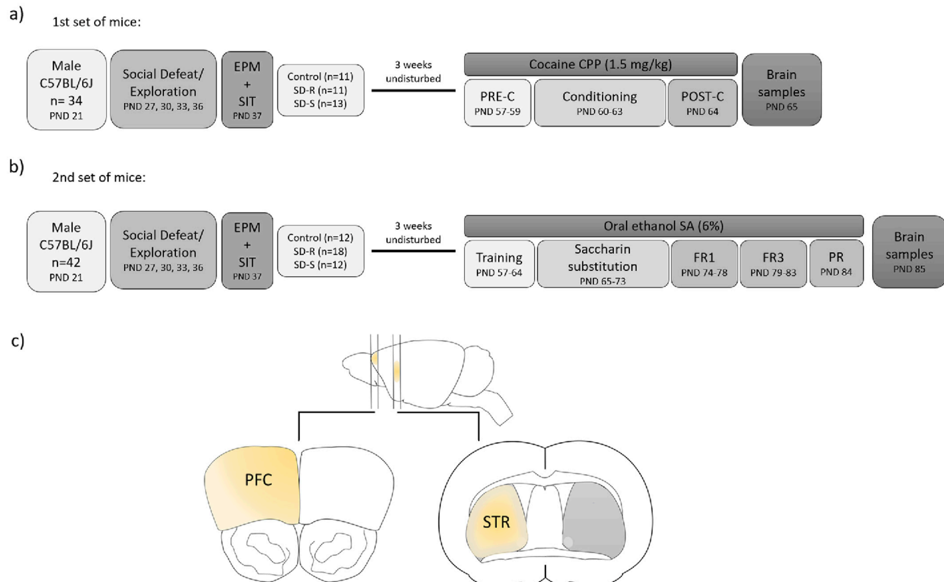

Fig. 1. Experimental design. Experimental protocol of the (a) first and (b) second sets of mice. (c) Diagram of the areas selected for immunoassay analysis. PFC prefrontal cortex; STR striatum.

that the probe mouse used in the social interaction test resembles the aggressor, and that this could foster social aversion, this is unlikely, since previous experiments demonstrate similar amounts of social investigation, irrespective of the strain used (i.e., C57BL/6; Berton et al., 2006). Before each session, the arena was cleaned with 5% alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Arena occupancy during object and social sessions was determined using the mice's horizontal position, controlled by commercial video tracking software (EthoVision XT 11, Noldus). Conventional measures of arena occupancy, such as time spent in the interaction zone and corners, were quantified. The former is commonly used as social preference-avoidance score and is calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restrain cage. Corners were defined as two squares of similar areas on the opposite wall of the arena. Social withdrawal ratio is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, mice with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011).

2.4.4. *Conditioned place preference (CPP)*

For place conditioning, we employed eight identical Plexiglas boxes with two compartments of equal size (30.7 × 31.5 × 34.5 cm high) separated by a gray central area (13.8 × 31.5 × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the position of the mice and their crossings from one compartment to the other to be recorded. The equipment was controlled by three computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

Place conditioning, consisting of three phases, was carried out during the dark cycle following a procedure that is unbiased in terms of initial spontaneous preference (Manzanedo et al., 2001). During the first phase -preconditioning (Pre-C)- mice were allowed access to both compartments of the apparatus for 900 s per day on 3 consecutive days. On day 3, the time spent in each compartment was recorded. Mice showing a strong unconditioned aversion (<33% of session time; i.e. 250 s) or preference (>67% of the session time; i.e. 650 s) for any compartment were discarded from the rest of the study. The ANOVA showed no significant differences between the time spent in the drug-paired and vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), which lasted 4 days, mice were conditioned with 1.5 mg/kg cocaine or saline. During this phase, half of the mice in each group received the drug or vehicle in one compartment, while the other half received it in the other compartment. An injection of physiological saline was administered before confining the mice to the vehicle-paired compartment for 30 min. After an interval of 4 h, the mice received cocaine immediately prior to confinement in the drug-paired compartment for a further 30 min. The central area was made inaccessible by guillotine doors during conditioning. The dose of cocaine used during the conditioning phase was a subthreshold dose (1.5 mg/kg, proven to be ineffective in controls) in order to evaluate increased sensitivity to the conditioned rewarding effects of cocaine. In the third phase -post-conditioning (Post-C)-, which took place on day 8, the guillotine doors separating the two compartments were removed, and the time spent in each compartment by the untreated mice during a 900 s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C and Pre-C tests is a measure of the degree of conditioning induced by the drug (conditioning score). If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates an aversion.

2.4.5. *Oral ethanol self-administration*

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA administration was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). Software package (Cibertec, SA, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 36 μl of fluid combined with a 0.5 s stimulus light and a 0.5 s buzzer beep, which was followed by a 6 s time-out period. Inactive nose-pokes did not produce any consequence.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, mice underwent an experiment carried out in three phases: training, saccharin substitution and 6% ethanol consumption.

2.4.5.1. *Training phase (7 days).* Two days before the initiation of the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and the food allotment was provided 1 h before the session to increase the motivation for active nose-poking. During the subsequent three days, water was provided ad libitum, except during the 1 h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and for the remainder of the experiment, food access was provided for 1 h after the end of each daily session and water was available ad libitum to avoid ethanol consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to respond to the active nose-poke to receive 36 μl of 0.2% (*w/v*) saccharin reinforcement.

2.4.5.2. *Saccharin substitution (10 days).* The saccharin concentration was gradually decreased as the ethanol concentration was gradually increased (Roberts et al., 2001; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% ethanol; 0.10% Sac −4% ethanol; 0.05% Sac −6% ethanol).

2.4.5.3. *6% ethanol consumption (11 days).* The aim of the last phase was to evaluate the number of active nose-poke responses, the 6% ethanol (w/v) intake and the motivation to drink. This phase began 38 days after the last SD. After each session, the alcohol that remained in the receptacle was collected and measured with a micropipette. To achieve this goal, during the last phase, the number of active responses and ethanol consumption (μl) were measured under a fixed ratio 1 (FR1) for 5 daily consecutive sessions, a fixed ratio 3 (FR3) (mice had to respond three times on the active nose-poke to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point (BP) for each mouse (the maximum number of nose-pokes each mouse is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1–2–3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the BP was calculated for each mouse as the maximum number of consecutive responses performed to achieve one reinforcement according to the previous scale. For example, if a mouse activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the BP value for this mouse would be 40. All the sessions lasted one hour, except the PR session, which lasted two hours.

2.4.6. *Immunoassay analysis (ELISA)*

Samples from the striatum and the PFC were obtained 24 h after

cocaine CPP and oral ethanol SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed, and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Heffner et al., 1980; Franklin and Paxinos, 2008, see Fig. 1c). The striatum and PFC were then kept in dry ice until storage at −80 °C. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na_3VO_4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

Some mice were discarded after measuring concentrations by ELISA due to a lack of signaling, and a few others were considered outliers.

2.5. Statistical analysis

Mice had been previously classified into resilient and susceptible groups based on their social withdrawal ratios. The social withdrawal ratio is calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends on the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, mice with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). No statistics were needed to identify separate groups of defeated mice and the criteria described in the statistical analysis section were sufficient to establish separate groups. The data of the time that the experimental mice and their aggressive opponents spent engaged in different behavioral categories during the SD episodes were compared by means of a mixed two-way ANOVA with one between-subject variable Stress, with two levels (Resilient and Susceptible); and one within subject variable Days, with two levels (1st and 4th SD). To evaluate the CPP induced by 1.5 mg/kg of cocaine, the conditioning scores were analyzed with a one-way ANOVA with a between-subjects variable –Stress, with three levels (Control, SD-R and SD-S). To analyze the acquisition of ethanol SA, a two-way ANOVA was performed with one between-subjects variable –Stress with three levels (Control, SD-R and SD-S)– and a within-subjects variable –Days, with five levels of FR1 or FR3. The effects of SD and treatment on BP values and ethanol consumption during PR was analyzed by a two-way ANOVA, with one between-subjects variable –Stress. The data of the CX3CL1 and IL-6 levels, as well as the EPM, were analyzed using a one-way ANOVA with one between-subjects variable –Stress, with three levels (Control, Resilient and Susceptible). For the data of the ethological analyses of SD and the EPM, all mice were analyzed together (1st and 2nd set) as the encounter, or the test occurred before the initiation of the cocaine CPP or the oral ethanol SA.

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. Statistical analyses were performed using SPSS Statistics (v.26; IBM, NY, USA) for behavioral data and GraphPad Prism (v8; GraphPad Software Inc., CA, USA) for graph design. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Classification between susceptible and resilient mice according to their social withdrawal ratios

In the first set of experimental mice (Fig. 2a), the Control group ($n = 11$) showed a mean social interaction ratio >1. Of 24 SD mice, 54% had interaction ratio <1 and 46% showed a ratio equal to or higher than 1. We classified mice with an interaction ratio <1 as SD-S mice ($n = 13$) and those with an interaction ratio greater than or equal to one as SD-R mice (n = 11).

In the second set of experimental mice (Fig. 2b), the Control group ($n = 12$) showed a mean ratio higher than 1. In the SD group of mice ($n = 30$), 40% showed a ratio under 1, which classifies them as susceptible (SD-S) mice (n = 12), and the remaining 60% showed a ratio equal to or higher than 1, which classifies them as resilient (SD-R) mice ($n = 18$).

3.2. All defeated adolescent mice increased passive-reactive coping during the 4th social defeat

The ANOVA for the time employed in Avoidance/Flee or Submissive/Defensive behaviors (Table 1) by defeated mice divided into resilient or susceptible according to their SIT scores showed an effect of the variable Days [F (1,52) = 16.5; $p < 0.001$] and [F (1,52) = 9.1; $p < 0.001$]. Likewise, the ANOVA for the latency to show Avoidance/Flee or Submissive/Defensive behaviors for the first time revealed an effect of the variable Days [F (1,52) = 16.2; $p < 0.001$] and [F (1,52) = 25.9; $p < 0.001$]. Defeated adolescent mice, classified as either resilient or susceptible, increased the time spent in these behaviors and were quicker to show them during the 4th social defeat ($p < 0.01$ for time spent in Submissive/Defensive behaviors and $p < 0.001$ for the rest of comparisons). The lack of differences depending on the SIT means that susceptible and resilient mice cope similarly with social defeat stress.

The ANOVAs for the time employed in Attack or Threat behaviors (Table 1) by resident mice showed an effect of the variable Days [F (1,52) = 16.9; $p < 0.001$] and [F (1,52) = 8.1; $p < 0.01$]. Resident mice decreased the time spent in these behaviors during the 4th SD compared to the 1st SD ($p < 0.001$ for time spent in Attack behavior and $p < 0.01$ for the time spent in Threat behavior). The ANOVAs for the latency to perform the first Attack revealed an effect of the interaction Days x Group [F (1,52) = 4.9; $p < 0.05$] and an effect of the variable Days [F (1,52) = 6.7; $p < 0.05$] for the latency to perform the first Threat behavior. Resident mice threatened faster in the 4th SD in comparison to the 1st SD in all groups ($p < 0.05$). However, resident mice attacked faster during the 4th SD only when attacking the SD-S group ($p < 0.001$).

3.3. Social defeat during adolescence induced anxiogenic effects in resilient mice

One outlier in time spent in the closed arms was removed from the Control group, along with two outliers in time spent in the open arms and in percentage of time in the open arms, and one outlier in the number of entries to the open arms, in total entries and in percentage of entries to the open arms in the SD-S group.

The data of the EPM test are presented in Fig. 3. The ANOVA of the time spent in closed arms [F (2,72) = 4.5; $p = 0.014$]; time spent in the open arms [F (2,72) = 3.9; $p = 0.023$]; percentage of time spent in the open arms [F (2,72) = 3.5; $p = 0.034$]; number of entries into the open arms [(2,73) = 3.6; $p = 0.031$], and percentage of entries into the open arms [(2,73) = 4.4; $p = 0.015$] revealed a significant effect of the variable Stress. Post-hoc analyses showed that resilient defeated mice

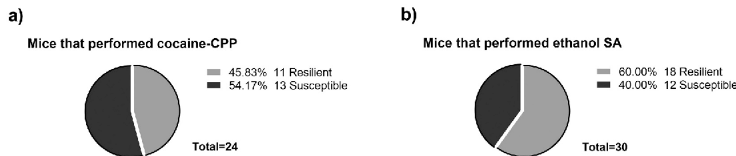

Fig. 2. Percentages of resilient and susceptible mice among groups of mice defeated during adolescence in the two experimental sets. The pie chart represents the percentage of resilient vs susceptible mice after social withdrawal ratio evaluation in the SIT in a) defeated mice that performed cocaine-CPP and b) defeated mice that performed ethanol SA paradigm.

Table 1
Coping behavior of the intruder mice during SD.

			SD-R		SD-S	
			1st SD	4th SD	1st SD	4th SD
Intruder mice	Submissive/ Deffensive	Time (s)	35 ± 4	50 ± 4 **	39 ± 4	52 ± 5 **
		Latency (s)	30 ± 6	11 ± 2 ***	40 ± 8	5 ± 1 ***
	Avoidance/ Flee	Time (s)	74 ± 7	116 ± 12 ***	72 ± 8	101 ± 15 ***
		Latency (s)	17 ± 3	7 ± 2 ***	26 ± 7	4 ± 1 ***
Resident mice	Attack	Time (s)	61 ± 5	38 ± 4 ***	60 ± 6	44 ± 4 ***
		Latency (s)	21 ± 3	19 ± 5	29 ± 6	9 ± 2 # # #
	Threat	Time (s)	31 ± 5	17 ± 2 **	30 ± 5	26 ± 3 **
		Latency (s)	23 ± 5	17 ± 5 *	33 ± 10	10 ± 2 *

Results are presented as mean values ±SEM. Mice were divided into resilient (*n* 29) and susceptible (*n* 25) depending on their SIT scores. Bonferroni post-hoc test *p < 0.05, **p < 0.01, ****p* < 0.001 significant difference compared to the 1st SD. ###p < 0.001 significant difference compared to the 1st SD in SD-S group.

according to their SIT scores spent more time in the closed arms, and less time and a lower percentage of time in the open arms than control non-stressed mice ($p < 0.05$ in all cases). Moreover, a lower number and percentage of entries into the open arms was registered in resilient defeated mice ($p < 0.05$ in all cases) with respect to the control group.

3.4. *Mice considered resilient according to their SIT scores developed a preference for cocaine-induced CPP*

The ANOVA for the time spent in the drug-paired compartment (Fig. 4a) showed a significant effect of the variable Days [F(1, 37) =5.2, $p = 0.030$], and the interaction Days x Stress [F(1,31) =3.3, $p = 0.05$]. Likewise, the ANOVA of the Conditioning Score (Fig. 4b) showed an effect of the variable Stress [F(2,31) = 4.7; $p < 0.016$]. Only resilient mice according to their SIT scores developed a preference for this cocaine dose and a significant increase in the time spent in the drug-paired compartment during the Post-C test was observed (p ⟨0,01). Consequently, higher conditioning scores were observed in the resilient mice than control mice ($p < 0.01$).

In addition, the analysis of the differences between the drug-paired and vehicle-paired compartments during the Pre-C and Post-C phases can be found in Table A of the supplementary material.

3.5. *Increased neuroinflammatory response observed following cocaine-induced CPP in susceptible mice compared to control mice*

The number of samples in each group was 10 for IL-6 and between 11 and 13 for CX3CL1. For IL6 determination, we lost 2 samples in the striatum of the control group (outliers), and another 2 in the striatum of the SD-R group (one outlier and one due to lack of signaling).

The ANOVA for the Il-6 levels in the striatum (Fig. 5a) showed a significant effect of the variable Stress [F(2,26) = 3.9; $p < 0.034$]. A higher concentration of Il-6 was observed in the striatum of susceptible mice according to their SIT scores compared to non-stressed control mice ($p < 0.05$). No differences were observed in the cortex (Fig. 5b).

With respect to fractalkine or CX3CL1 levels, although there were no differences in the striatum (Fig. 5c), a higher concentration was observed in the PFC of both susceptible and resistant mice [F(2,35) = 4.5; $p < 0.019$] compared to non-stressed controls ($p < 0.05$ in both cases) (Fig. 5d).

3.6. *Resilient mice showed higher ethanol intake than control mice*

No differences were found in the active responses or between the different groups during the training or substitution phases, demonstrating that SD did not induce any learning deficits. No differences were found in the body weight of the mice during the FR1, FR3 and PR schedules. Analyses of the acquisition and substitution phases of SA and body weights during the FR1, FR3 and PR schedules can be found on the supplementary material.

The ANOVA for the number of active responses during FR1 schedule (Fig. 6a) did not reveal any significant effects, although there was a tendency toward a Stress effect [F(2,39) = 3.1; $p = 0.058$). Resilient mice tended to perform more active responses than controls ($p < 0.058$). With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Stress [F(2,39) = 4.5; $p = 0.018$) (Fig. 6b). The post-hoc comparison showed that the resilient mice consumed ethanol at higher rates than the control ($p < 0.05$) and susceptible mice ($p < 0.01$).

During the FR3 schedule, the ANOVA of the number of active responses (Fig. 6a), the ANOVA revealed a significant effect of the variable Days [F(4,156) = 4.4; $p < 0.002$] and Stress [F(2,39) = 6.3; $p < 0.004$]. All mice made significantly more active responses on days 9 and 10 than on day 6 ($p < 0.01$ for day 9, and p < 0,05 for day 10). Moreover, resilient mice performed more active responses during the FR3 than non-stressed controls ($p < 0.01$). With respect to the ethanol consumption, the ANOVA revealed a significant effect of the variable Days [F(4,156) = 16.4; $p < 0.001$] and Stress [F(2,39) = 6.3; $p < 0.004$] (Fig. 6b). All mice consumed significantly more ethanol on days 7, 8, 9 and 10 than on day 6 ($p < 0.001$ in all cases). Moreover, resilient mice consumed more ethanol during the FR3 than non-stressed controls ($p < 0.01$).

During the PR, the ANOVA for the BP values of ethanol SA revealed a significant effect of the variable Stress [F(2,39) = 4.9; $p < 0.012$] (Fig. 6c). The post-hoc comparison showed that resilient mice achieved higher BP values than the control group ($p < 0.01$). The ANOVA for ethanol consumption during PR did not reveal a significant effect of the variable Stress (Fig. 6d).

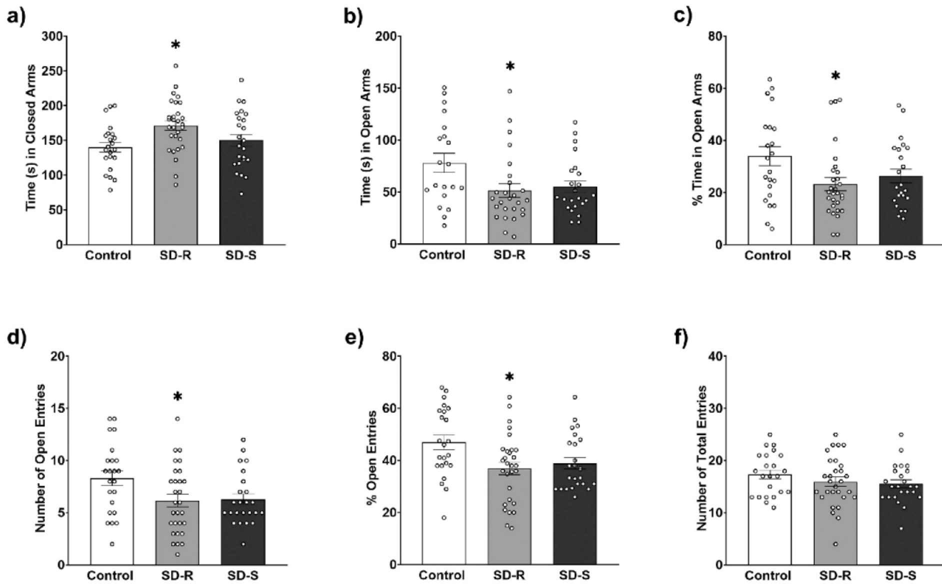

Fig. 3. Long term effects of SD on anxiety-like behavior. Bars represent the mean (± SEM) of (a) time in closed arms in seconds, (b) time in open arms in seconds, (c) percentage of time in open arms, (d) number of open entries, (e) percentage of open entries and (f) number of total entries. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

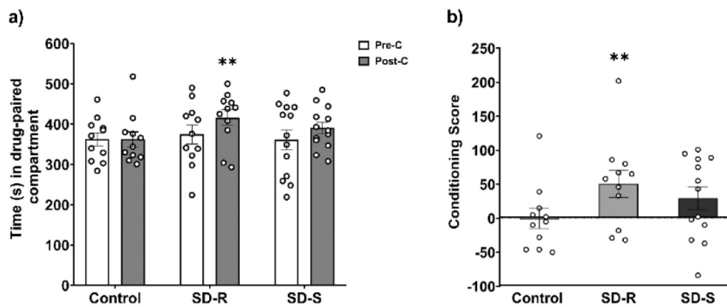

Fig. 4. Resilient mice showed higher preference in cocaine-induced CPP than susceptible mice. Effect of adolescent SD on cocaine-induced CPP. Mice were divided into Control (n 11); Resilient (n 11) and Susceptible (n 13). Defeated mice were characterized as resilient or susceptible depending on their SIT scores. a) The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (Pre—C) (white bars) and after conditioning sessions in the post-conditioning test (Post-C) (gray bars), during which CPP was induced with 1 mg/kg of cocaine. b) The bars represent the conditioning score (difference in seconds between the time spent in the drug-paired compartment after the conditioning sessions and that spent in the same compartment during Pre—C). **$p < 0.01$ significant difference in the time spent in the drug-paired compartment vs Pre-C session or with respect to the control group.

3.7. Increased neuroinflammatory response observed in susceptible mice compared to control mice following oral ethanol self-administration

The number of samples in each group was between 11 and 13 for IL-6 and between 12 and 14 for CX3CL1. For CX3CL1 determination, we lost 3 samples in the SD-R group (three outliers).

The ANOVA for the Il-6 levels in the striatum (Fig. 7a) showed a significant effect of the variable Stress [$F(2,36) = 3.9$; $p < 0.023$]. A higher concentration of Il-6 was observed in the striatum of susceptible mice according to their SIT scores compared to the non-stressed control group ($p < 0.05$). No differences were observed in the PFC (Fig. 7b).

With respect to fractalkine or CX3CL1 levels in the striatum (Fig. 7c) the ANOVA showed a significant effect of the variable Stress [$F(2,33) = 3.8$; $p < 0.03$], as significantly elevated levels were observed in the

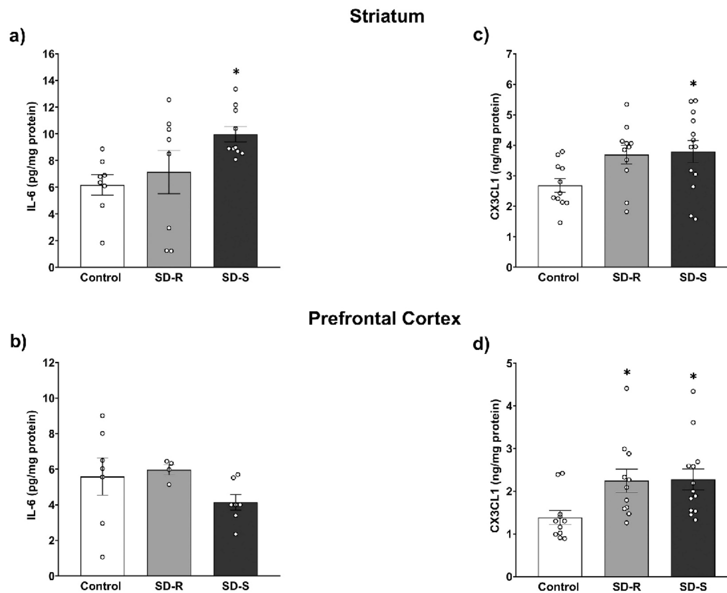

Fig. 5. Susceptible mice showed higher neuroinflammatory markers after cocaine-induced CPP than resilient mice. Effect of repeated SD on striatal and cortical levels of IL-6 and CX3CL1. Bars represent the mean (± SEM) of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6. Similarly, bars represent the mean (± SEM) of the striatal (b) and cortical (d) levels (in ng/mg) of the pro-inflammatory chemokine CX3CL1 and the vertical lines ± SEM. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

susceptible mice compared to the control group ($p < 0.03$). No differences were observed in the PFC (Fig. 7d).

4. Discussion

A recent meta-analysis reported that 62.5% of individuals start to show signs of mental disorders by the age of 25, with a peak at 14.5 years of age (Solmi et al., 2021). Although there is strong evidence linking bullying and later mental illness (McKay et al., 2021), only few adolescents suffering from these traumatic events will develop psychiatric disorders (Dumont and Provost, 1999; Aarestad et al., 2021). Studies suggest that a correct reaction of the body is crucial for an adaptive response to the environment and to avoid stress-related deficits (Cathomas et al., 2019; Dutcher and Creswell, 2018). We already know that a percentage of adult rodents exposed to SD will show a resilient phenotype to social avoidance and to the increase in the rewarding effects of cocaine and ethanol intake (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). However, few studies have been performed to know whether stressed adolescent mice will show a consistent resilient phenotype, as adult mice do. The fact that the developmental process of resilience seems to strengthen over time, together with the increased salient value of social interactions in adolescent mice, indicates that the study of the resilient phenotype in adolescent defeated rodents is highly needed (Sheth et al., 2017; Malhi et al., 2019).

Our results showed that mice defeated during adolescence showed marked differences in their resilient response in comparison with the experience of SD during adulthood. Resilience to the detrimental effects of experiencing SD during adolescence did not develop as a unique phenotype. Mice resilient to depressive-like behavior showed an increased anxiogenic behavior, a higher response to cocaine and higher ethanol intake compared to the control group. However, the increased neuroinflammatory response was present mainly in the mice susceptible to depressive-like behaviors compared to the control group, despite showing a normal response to cocaine and ethanol.

4.1. *Adolescent defeated mice showed behavioral flexibility coping with stress*

We know that passive coping strategies in response to social stress are associated with more pronounced physiological effects and psychopathology (Hawley et al., 2010; Russo et al., 2012; Wood and Bhatnagar, 2015). Submissive and immobile behavior are considered passive-reactive coping strategies. Meanwhile, active coping is characterized by longer latency to display the defeat posture, fight-back or active escape (Koolhaas et al., 2007; Wood et al., 2010).

We have previously reported that adult mice resilient to the increase in cocaine reward display fewer flee/avoidance and submissive/defensive behaviors during SD than those categorized as susceptible according to their SIT scores (Ballestín et al., 2021; Ródenas-González et al., 2021). In contrast with these results, there were no differences between resilient and susceptible adolescent mice in the way of coping with SD. All defeated adolescent mice showed an increase in the time spent in defensive or flee behaviors in the fourth SD and presented shorter latencies to show these behaviors. The increased time in these behaviors indicates behavioral flexibility to the inescapable SD experience and is characteristically observed only in resilient adult mice. Behavioral flexibility has been associated with emotional resilience, less reactivity of the HPA axis, and increased neuroplasticity (Hawley et al., 2010;

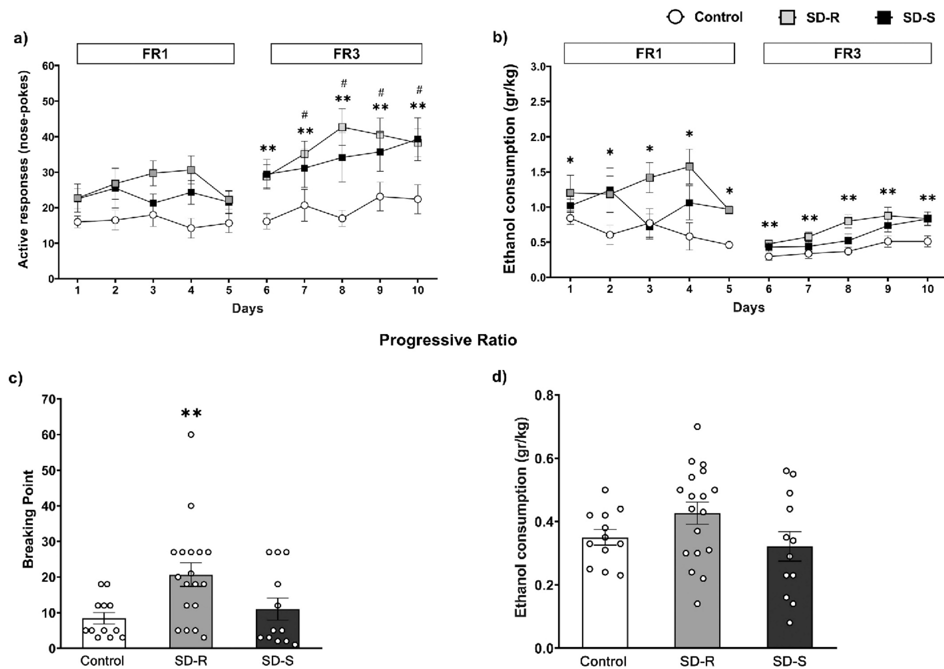

Fig. 6. Resilient mice showed higher ethanol intake than susceptible mice. Mice were divided into Control (n 12); Resilient (n 18) and Susceptible (n 12). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses; (b) the volume of 6% ethanol consumption during FR1 and FR3; (c) the BP values; and (d) the volume of 6% ethanol consumption during PR. *$p < 0.05$, **$p < 0.01$ significant difference with respect to controls; #$p < 0.05$ significant difference with respect to day 6.

Lambert et al., 2014). Therefore, the adolescent response to SD indicates an adequate adaptation to stress.

Despite this adaptive response to SD, the percentage of resilient and susceptible adolescent mice classified according to the SIT, which evaluates depressive-like behaviors, is similar to that observed in adult defeated mice (Giménez-Gómez et al., 2021), with 53% of adolescent defeated mice showing a resilient phenotype to depressive-like behaviors. Albeit with slightly methodological differences, this percentage was also observed in the study of Alves-dos-Santos et al. (2020) and Vassilev et al. (2021) with adolescent defeated mice.

One limitation of the present study is the fact that resilient and susceptible mice were housed together in the same cage throughout the entire procedure. Mice were housed in groups of 4 when they arrived at the laboratory on PND 21 and resilient or susceptible phenotypes to depressive-like behaviors were evaluated 24 h after the last SD on PND 37. Housing resilient and susceptible mice together at that moment would have implied a deeply stressful hierarchical reorganization in each cage, which could affect the behavioral and biochemical results. Therefore, housing resilient and susceptible mice in the same cage must be taken into consideration as a potential variable that could affect the results obtained. Another variable to take into account is the fact that injuries derived from confrontations within the home cage could affect neuroinflammatory markers. However, we find this possibility very unlikely, as the condition of the mice was monitored daily and injuries among adolescent C57BL/6 J strain mice were not observed.

4.2. Anxiogenic response in resilient mice

It has been extensively established that SD induces an acute anxiogenic response (Albrechet-Souza et al., 2017; Ferrer-Pérez et al., 2019; Macedo et al., 2018), an effect that is lacking in resilient mice. We have previously observed that adult mice defeated during adulthood that present a resilient phenotype to depressive-like behaviors and the rewarding effects of cocaine did not present this anxiety increase. In addition, all the environmental or pharmacological treatments that increase the percentage of resilient mice to SD also increase the percentage of mice that did not experience an anxiogenic response (Giménez-Gómez et al., 2021). However, when the SD took place during adolescence, the results are controversial and the experimental protocol used to induce social stress and the time proximity between behavioral tests and stress play a key role in the response observed. Several reports confirmed that after 3 weeks of the last SD there were no observable effects in the EPM in defeated adolescent mice (Rodríguez-Arias et al., 2016; Watt et al., 2009). Alves-dos-Santos et al. (2020) did not observe anxiety-like behaviors in the EPM test when compared to control mice. In that case, the EPM took place in the last days of 10 sessions of chronic social defeat stress and mice were isolated during the entire procedure, which could affect the response. However, in agreement with Iñiguez et al. (2014), we observed that all defeated mice showed an increase in anxiogenic behaviors 24 h after the last encounter, but only resilient mice to depressive-like behavior spent less time and percentage of time in the open arms. Different results were observed by Vassilev et al. (2021) with

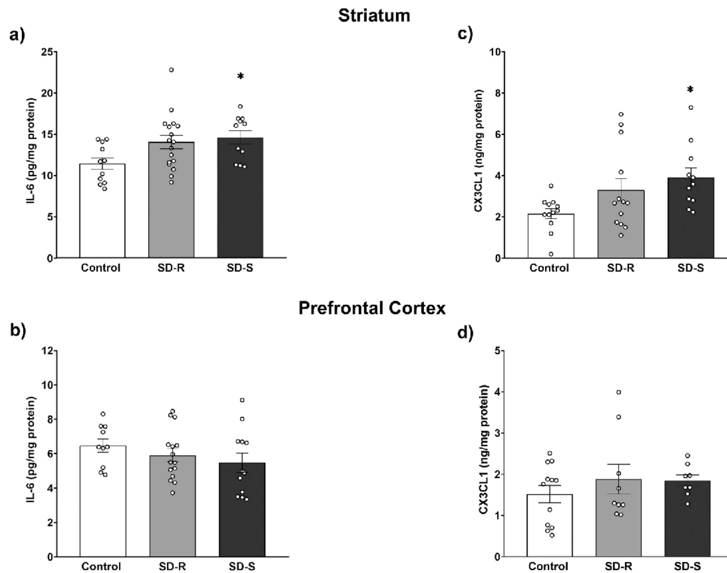

Fig. 7. Susceptible mice showed higher neuroinflammatory markers after oral ethanol self-administration than resilient mice. Bars represent the mean (± SEM) of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6. Similarly, bars represent the mean (± SEM) of the striatal (b) and cortical (d) levels (in ng/mg) of the pro-inflammatory chemokine CX3CL1 and the vertical lines ± SEM. Bonferroni post-hoc test * $p < 0.05$, significant difference compared to the control group.

no increased anxiety observed in susceptible mice, but surprisingly, in resistant mice an anxiolytic response was observed with an increase in the time spent in the open arms, which the authors suggest may be due to higher propensity for risk-taking-like behaviors in resilient adolescent mice.

Therefore, our results indicate that, differently from the adult response to SD, adolescent mice developed a partial resilient response to anxiety and this was not associated with depressive-like behaviors.

4.3. *Increased response to cocaine and ethanol in resilient mice compared to the control group*

As we have previously reported for adult mice, a subset of defeated adolescent mice developed a preference for cocaine (Ballestín et al., 2021; Giménez-Gómez et al., 2021). However, different from adult defeated mice, adolescent mice resilient to depressive-like behaviors according to their SIT scores showed a preference for this dose of cocaine. On the other hand, susceptible mice showing social avoidance did not develop a preference for cocaine. However, we should note that a significant preference for the drug-paired compartment compared to the vehicle-paired compartment was observed during the post-conditioning phase in all defeated mice (see Table A in the Supplementary Material). Although SD-S mice did not develop a preference or an increase in the conditioning score, social defeat seems to exert some effect on cocaine preference compared to vehicle administration.

In line with the results observed with cocaine, resilient adolescent mice according to their SIT scores showed higher ethanol intake than control mice. Once again, defeated adolescent mice behave differently from adults. Resilient adult mice according to their SIT scores showed a complete phenotype with no social avoidance and drinking a similar amount of ethanol as non-stressed control mice. On the other hand, defeated adult mice susceptible to social avoidance presented a significantly higher ethanol intake (Reguilón et al., 2021b).

In our defeated adolescent mice, the analysis of the two phenotypes (depressive-like behaviors and response to cocaine or ethanol) showed that, among mice resilient to depressive-like behaviors (with SIT scores superior to 1), 45% did not develop a preference for cocaine (see Table 2). Based on previous studies with this strain of mice, we considered that an increase similar or superior to 60 s was the minimum increase necessary to develop a preference (Ballestín et al., 2021; Giménez-Gómez et al., 2021). Therefore, only 21% of the defeated mice showed resilience to both phenotypes. Similar results were also obtained in the 2nd experiment, with only 33% of resilient mice depending on the SIT drinking ethanol similarly to control mice. In that case, mice that drank ethanol more than two standard deviations from the control mean were considered susceptible. As in the 1st experiment, 20% of the defeated mice showed a complete resilient phenotype. Therefore, there is an inconsistent development of resilience between depressive-like

Table 2
Percentage of resilient and susceptible mice.

	SWR	Cocaine preference		Fully Resilient /Susceptible
Resilient	46%	>60 s (susceptible)	55%	21%
		<60 s (resilient)	45%	
Susceptible	54%	>60 s (susceptible)	38%	21%
		<60 s (resilient)	62%	
	SWR	Ethanol intake (ml/g)		Fully Resilient /Susceptible
Resilient	60%	>7 (susceptible)	67%	20%
		<7 (resilient)	33%	
Susceptible	40%	>7 (susceptible)	58%	23%
		<7 (resilient)	42%	

behaviors and response to drug reward.

4.4. Increased neuroinflammatory response in susceptible mice compared to the control group

Psychological stress induces a series of neuroimmune reactions involving a bidirectional brain-immune signaling that affects mood and behavior (Wohleb et al., 2015). Long-term increments in proinflammatory cytokines such as IL-6 levels after repeated SD have been described in several mice brain areas (Ferrer-Pérez et al., 2018; Montagud-Romero et al., 2020; Montagud-Romero et al., 2021; Reguilón et al., 2021b). In adult mice, resilience to depressive-like behaviors and the increase in cocaine or ethanol reward are associated with a minor neuroinflammatory response. In susceptible mice, an increase in IL-6 levels was observed compared to those of controls or resistant mice shortly after SD or after cocaine or oral ethanol SA (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021b). These results have also been confirmed in adolescent defeated mice. Although no increased response to cocaine or ethanol was observed in the susceptible mice, an increase in IL-6 levels was observed in the striatum after cocaine-induced CPP and oral ethanol SA, compared to the control group.

With respect to the chemokines response, SD induced changes in CX3CL1 or fractalkine, which seems to depend on the mouse strain used, probably due to their different sensitivity to social stress. Although SD induces increases of striatal CX3CL1 levels in OF1 mice (which are highly territorial) (Reguilón et al., 2020; Reguilón et al., 2021a), in C57BL/6 mice the opposite result was found. Both resilient and susceptible mice, decreases in striatal fractalkine levels were observed immediately after the last SD and even after cocaine-induced CPP (Ballestín et al., 2021). Moreover, after oral ethanol SA, decreases were only observed in susceptible mice (Reguilón et al., 2021b). Surprisingly, compared with the control group, increased levels of CX3CL1 were observed in the striatum of adolescent susceptible C57BL/6 mice following exposure to cocaine or ethanol, and cortical levels were increased in all adolescent defeated mice only following exposure to cocaine. Only few studies have evaluated this chemokine in adolescent mice, but a recent study by Liu et al. (2020b) found increased CX3CL1 expression in the hippocampus of adolescent mice exposed to nicotine during gestation and lactation.

Exposure to social stress during early adolescence produced a permanent alteration of microglia morphology and the induction of an inflammatory episode in the ventral tegmental area (VTA) (Lo Iacono et al., 2018). This inflammatory episode altered the functionality of dopaminergic neurotransmission in the VTA following exposure to a cocaine-CPP in adulthood. The authors of this study concluded that social stress during early life sensitizes the reward pathway and the immune response. In line with this, the inflammatory responses observed in our study may be potentiated by the profound alteration of the immune system produced by social defeat in combination with subsequent exposure to substances of abuse.

4.5. Characteristic development of resilience to SD in adolescent mice

In these experiments, we demonstrated that experiencing SD during adolescence presents specific characteristics. In agreement with the few studies performed in this area, defeated adolescent mice did not develop a general resilient/susceptible phenotype. There is no correlation between the resilience to social avoidance and the increased response to cocaine and ethanol or the neuroinflammatory response. Although, differently from adults, all adolescent mice presented an adaptive coping mechanism with stress, the percentage of resilient/susceptible mice after SD is comparable to that observed in adult mice. Increased anxiogenic behavior, preference for the cocaine-paired compartment or ethanol intake were observed in mice resilient to the development of social avoidance. However, resilience to social avoidance correlated with a minor neuroinflammatory response. These results indicate that the age of exposure to SD affects the development of resilience.

In line with our results, Alves-dos-Santos et al. (2020) observed that defeated adolescents resilient to anhedonia or social avoidance were the most affected mice in terms of both endocrine/physiological outcomes (body weight gain and corticosterone response). Likewise, Vassilev et al. (2021) observed that, in adolescence, SD produces inhibitory control impairment independently from social avoidance. As with ours, all these studies have been performed only in males, which is an important limitation of the present investigation. Marked sex differences in stress responses have been reported in adult rodents and humans (for reviews, see Hodes and Epperson, 2019; and Wellman et al., 2018), but limited data in female rodents during adolescence are available.

Resilience should be considered an active process, which affects both passive and active strategies, in order to achieve the highest adaptation to stress (Russo et al., 2012). Responses on each particular system may develop differently after exposure to stress (Smith, 2019). Our results suggest that SD during adolescence leads to an addiction-prone phenotype in some mice, which manifests itself as resilient during the SIT and presents a normalized neuroinflammatory response.

The response to drugs of abuse is based on their rewarding properties, which depend on the function of the mesocorticolimbic dopaminergic system. Vassilev et al. (2021) observed that SD during adolescence, but not in adulthood, dysregulates the Netrin-1/DCC pathway in the VTA and the nucleus accumbens, which induced changes in dopamine (DA) connectivity in the PFC. These authors observed that, although a reduction in VTA DCC expression was observed in all defeated mice, ectopic growth of mesolimbic DA axons was observed into the medial PFC of resistant mice. This specific adaptation on the dopaminergic system could be related to the increased response to cocaine and ethanol observed in resilient mice.

5. Conclusions

Adolescence is a critical developmental period for later mental illness, and an important period in which to focus intervention strategies. More studies are needed in order to fully evaluate the relationship between bullying and substance use disorders. A recent study showed a bidirectional correlation indicating that individuals who engaged in substance use were more likely to perpetrate cyber aggression than those who did not, a result that suggests a strong relationship between substance use and bullying (Crane et al., 2021).

Our findings illustrate that, contrary to prior assumptions in adults, SD stress responses are more complex and singular in adolescents, and caution should be taken for the correct interpretation and translation of those phenotypes. The Social Interaction Test, considered a depressive-like phenotype, is currently used to classify mice into resilient or susceptible in order to study the neurobiology and molecular aspects of social stress (e.g. Russo et al., 2012). In adolescents, we should not assume that resilience to one phenotype equally develops for others, highlighting the concept of resilience as an active process affected by the person's age at the moment of the stress experience.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2022.110591.

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Ethical statement

The experimental protocol has been approved by an Institutional Review Committee for the use of animal subjects. Procedures involving mice and their care were conducted in conformity with national, regional and local laws and regulations, which are in accordance with European Community Council Directives 2010/63/UE regulating animal research and were approved by the local ethical committees. All the efforts were made to minimize animal suffering and to reduce the number of animals used.

CRediT authorship contribution statement

Marina D. Reguilón: Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization. **Raúl Ballestín:** Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization. **José Miñarro:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marta Rodríguez-Arias:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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