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DOCTORAL THESIS

Characterization and enhancement of resilience to the effects of social stress on the rewarding properties of cocaine in male mice.

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CERTIFICAN

Que la tesis doctoral presentada por Doña Claudia Calpe López, con el título "Characterization and enhancement of resilience to the effects of social stress on the rewarding properties of cocaine in male mice" ha sido realizada bajo su dirección y que tras haberla examinado hacen constar su autorización para que se realicen los trámites conducentes a su defensa.

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RESUMEN AMPLIO

El estrés es una respuesta fisiológica y adaptativa del organismo a las condiciones ambientales y cada individuo lo enfrenta de manera diferente. A corto plazo, puede ayudar a alcanzar objetivos y muy probablemente nos proporcione placer o *eustress* (de la palabra griega *eu*, que significa bueno) (Selye, 1975). Sin embargo, cuando el estrés se torna crónico y la respuesta hormonal al estrés se prolonga y persiste en el tiempo, aparecen síntomas nocivos para el organismo (Satsangi y Brugnoli, 2018; Sarjan y Yajurvedi, 2018). Pueden sobrevenir diversas dolencias físicas como dolor en diferentes partes del cuerpo, acidez estomacal, diarrea, así como el desarrollo de otras patologías como el síndrome del intestino irritable, reacciones cutáneas, fatiga, hipertensión, dificultades respiratorias y mareos, etc. (Bennett et al., 1998; Konturek et al., 2011; Liu et al., 2017). Además de las consecuencias sobre la salud física, numerosos estudios han demostrado la relación entre el estrés crónico, la función cognitiva y la salud mental (véase la revisión de Marin et al., 2011).

Nuestro entorno social es cambiante y complejo y demanda lidiar persistentemente con diferentes tipos de estresores por lo que puede afectar a nuestro equilibrio y bienestar (Pearlin, 2010; Pearlin et al., 2005). Está extensamente documentado que una de las principales fuentes de estrés emana de la interacción social (Tough et al., 2017; Sánchez-Salvador et al., 2021; Miller y Kirschbaum, 2019), es decir, de los problemas o peleas que surgen entre compañeros y superiores, con vecinos, entre los propios amigos o dentro del hogar. Por esta razón, el estrés social influye significativamente en la incidencia de diversas enfermedades mentales, incluyendo el desarrollo de conductas adictivas (Atrooz et al., 2019; Koo y Wohleb, 2021; Cattaneo y

Riva, 2016). La literatura científica muestra que en modelos animales las experiencias de estrés social inducen un incremento en la respuesta reforzante de las drogas y facilitan la reinstauración de la búsqueda de la droga tras periodos de abstinencia en paradigmas como el condicionamiento de la preferencia de lugar (CPL) (Montagud-Romero et al., 2018) o la autoadministración de drogas (Rodríguez-Arias et al., 2016, 2017). Las consecuencias del estrés se reflejan en diversos sistemas cerebrales, entre los que podemos destacar el sistema dopaminérgico mesolímbico (Tielbeek et al., 2018; Li et al., 2016; Vaessen et al., 2015; Bonapersona et al., 2018), en los que se observan cambios epigenéticos y neuroplásticos y alteraciones estructurales y funcionales a largo plazo (Turecki y Meaney, 2016; Montagud-Romero et al., 2016; Park et al., 2019).

Como ya hemos señalado, el estrés social crónico influye negativamente en todas las etapas del proceso adictivo o trastorno por consumo de sustancias (TCS), según la terminología del DSM-5. Es decir, el estrés facilita la adquisición y el mantenimiento del consumo de drogas, promueve la escalada (abuso) y, después de un período de abstinencia o desintoxicación, puede inducir la recaída en el consumo de drogas (George et al., 2012; Zorrilla et al., 2014; Koob y Schulkin, 2019*).* Así pues, al desarrollo del TCS contribuye de forma esencial el intento del individuo de limitar los estados afectivos negativos y los síntomas relacionados con el estrés durante la abstinencia a corto plazo o prolongada y, a su vez, estos periodos de abstinencia pueden en sí mismos promover una respuesta fisiológica y psicológica de estrés (Koob y Schulkin, 2019*).* De hecho, el TCS presenta una alta comorbilidad y prevalencia con el estrés crónico, el estrés traumático o el trastorno de estrés postraumático (TEPT) (Saunders et al., 2016; Tiet y Moos, 2021; Leconte et al., 2022). Desde una perspectiva neurocientífica, la

adicción es una enfermedad crónica caracterizada por la pérdida de control en el consumo de una sustancia adictiva y, por lo tanto, daña gravemente el funcionamiento humano en todas sus facetas (Zou et al., 2017). El origen es multifactorial, por lo que participan factores predisponentes tanto ambientales como biológicos *(*Karila, & Benyamina, 2019). En el contexto social, las experiencias estresantes se consideran factores de riesgo ambientales que interaccionan con los factores biológicos promoviendo un incremento en la vulnerabilidad a desarrollar un TCS (Cadet et al., 2016). Por todo ello, resulta fundamental desarrollar estrategias que permitan controlar el impacto del estrés crónico sobre la vulnerabilidad a desarrollar dicha enfermedad.

En este sentido, existe un interés creciente en los últimos años en el fenómeno de la resiliencia, entendida como la capacidad de un individuo para enfrentarse y superar un evento traumático o un período de dificultad (Earvolino-Ramirez, 2007; Garcia-Dia et al., 2013; Johnson et al., 2017; Babic et al., 2020). Popularmente hay varios términos para definir la resiliencia, pero todos tienen el mismo significado que incluye la capacidad de permanecer de pie después de la dificultad, la elasticidad, la firmeza o la robustez y la lucha. Ser resiliente sería como estar constituido de material flexible que vuelve a su forma original después de haber sufrido un daño externo. Desde un punto de vista científico, la resiliencia es la capacidad que presentan la mayoría de los individuos para mantener un funcionamiento psicológico y físico adaptativo, y evitar así la aparición de enfermedades mentales cuando están expuestos a altos niveles de estrés (Charney, 2004).

Lo opuesto a la resiliencia sería fundamentalmente la susceptibilidad a los problemas, pero también a los riesgos, así como una fuerte vulnerabilidad, sensibilidad y tristeza, que se agravan a través del estrés. La resiliencia en sí

representa la resiliencia mental. Se trata de encontrar la propia fuerza para afrontar las adversidades de la vida, las crisis más o menos fuertes, que a menudo afectan la propia integridad física o psicológica. De alguna manera la resiliencia sería equivalente a nuestro sistema inmunológico mental, es nuestro medio y nuestra herramienta para manejar despidos, separaciones, duelos, disputas y otros problemas sistémicos, así como los trastornos relacionados con el estrés, el agotamiento, la depresión, etc. La mejor parte de la resiliencia es que permite a las personas crecer a través de los desafíos y las prepara para los próximos potenciales contratiempos.

Hasta hace poco tiempo el estudio de la resiliencia ha sido primordialmente fenomenológico, pero en los últimos años se han comenzado a identificar las características psicológicas y biológicas de las personas resilientes, es decir aquellas que no desarrollan enfermedades mentales como la depresión o el TEPT tras la exposición al estrés. El concepto de resiliencia ha implicado un cambio de paradigma en los campos de la medicina y la psicología, ya que se centra en los factores que mantienen la salud y promueven el bienestar en lugar de centrarse en los factores de vulnerabilidad a la enfermedad. En el caso de la adicción a las drogas la mayoría de las investigaciones ha tenido como objetivo identificar los factores individuales y ambientales que aumentan la susceptibilidad de un sujeto a la drogadicción. La incorporación del estudio de la resiliencia, como un constructo complejo y multidimensional, nos permitirá desentrañar los rasgos neuroconductuales que confieren protección contra el desarrollo de un trastorno adictivo tras la exposición a eventos estresantes o traumáticos, así como los sustratos neurobiológicos subyacentes a esta resiliencia. Los estudios realizados en la última década sobre la neurobiología de la resiliencia a desarrollar trastornos mentales, aunque están todavía en una fase inicial, han permitido identificar

factores genéticos, epigenéticos, moleculares, neuroquímicos, psicológicos y ambientales que protegen a los individuos de desarrollar depresión o TEPT (Averill et al., 2018; Baratta y Maier, 2019; Osório et al., 2017; Calpe-López et al., 2022a; al'Absi, 2020). En la actualidad, la resiliencia se considera un proceso activo y dinámico que puede potenciarse para permitir que los individuos se adapten positivamente a un contexto estresante que, en otro caso, podría aumentar el riesgo de desarrollar un trastorno psiquiátrico. Esta concepción ha estimulado la realización de estudios centrados en factores de protección específicos y en cómo pueden manipularse los mecanismos neurobiológicos que subyacen a la resiliencia a desarrollar trastornos del estado de ánimo y de ansiedad (eje hipotálamo-pituitario-adrenal, GABA, serotonina, glutamato, dopamina, noradrenalina, acetilcolina, endocannabinoides, BDNF, hipocretina, NPY, galanina, etc.) para aumentar la resiliencia al estrés en individuos de alto riesgo y así prevenir el desarrollo de trastornos psiquiátricos relacionados con el estrés (Faye et al., 2018; Srinivasan et al., 2013; Averill et al., 2018; Albrecht et al., 2021; Stainton et al., 2019). Estos estudios sobre resiliencia, al igual que los que componen la presente tesis doctoral, pueden servir de base para la búsqueda de nuevas dianas terapéuticas para la adicción a las drogas.

Como ya se ha mencionado, tras la exposición al estrés, algunos humanos desarrollan un trastorno psicopatológico, como la depresión o la ansiedad, mientras que otros son resilientes a dichos efectos. Los modelos animales son necesarios para comprender los diferentes aspectos de la resiliencia humana, así como los sustratos neurobiológicos o conductuales que subyacen a la resiliencia a desarrollar trastornos mentales tras la exposición a estrés. Los trastornos mentales son complejos y multifactoriales y afectan a muchos aspectos de la vida humana; por tanto, ningún modelo

animal puede imitar la complejidad de los trastornos humanos. Sin embargo, los modelos animales son útiles para simular algunos de los síntomas psiquiátricos (Harris, 1989) o dimensiones conductuales que caracterizan un trastorno (Frazer y Morilak, 2005). Tras la exposición al estrés crónico, algunos animales desarrollan síntomas similares a los de la depresión y la ansiedad y otras alteraciones conductuales (animales susceptibles o vulnerables), mientras que otros muestran una clara resistencia a, al menos, algunas de las secuelas desadaptativas del estrés (animales resilientes). Además, los modelos animales también contribuyen a nuestra comprensión de los mecanismos que subyacen al desarrollo de la resiliencia, como los efectos terapéuticos de la inoculación del estrés (Ayash et al., 2020). De la misma forma, la investigación preclínica ayuda a comprender en mayor profundidad los efectos reforzantes de las drogas y, en última instancia, contribuye a mejorar el bienestar de las personas afectadas por un TCS. La mayoría de experimentos sobre resiliencia al estrés en animales experimentales utilizan el modelo denominado estrés por derrota social (DS) repetida o crónica. Este es un modelo con gran relevancia etológica, ya que como hemos comentado anteriormente, la forma más común de estrés experimentada por los seres humanos proviene de su ambiente social. Además, el modelo posee validez aparente para modelar la sintomatología de los trastornos relacionados con el estrés, como el TEPT y la depresión. En este modelo, tras exponer a los animales a la DS se realiza una prueba de interacción social observándose dos respuestas conductuales fenotípicas: evitación social prolongada (característica de los animales que desarrollan sintomatología de depresión o TEPT), o interacción social normal (característica de los animales resilientes). En nuestro laboratorio hemos demostrado también que la DS repetida es un modelo animal útil para estudiar

la influencia del estrés sobre los efectos reforzantes de drogas como la cocaína (Montagud-Romero et al., 2016, 2017; Rodríguez-Arias et al., 2017), el alcohol (García-Pardo et al., 2016; Rodríguez-Arias et al., 2016) y el MDMA (García-Pardo et al., 2015).

El principal foco de interés en la presente Tesis es estudiar la resiliencia a desarrollar un trastorno por consumo de cocaína tras la exposición a estrés. Para ello utilizamos modelos animales de estrés social (la DS repetida intermitente, DSRI) y de efectos reforzantes de las drogas (el CPL), ya que en estudios previos hemos demostrado que la exposición a DSRI incrementa los efectos reforzantes de la cocaína en el CPL (García-Pardo et al., 2019; Calpe-López et al., 2020). Nuestro objetivo es identificar a los animales resilientes a este efecto de la DSRI, caracterizar su comportamiento tras el estrés, estudiar si existe una relación con la resiliencia a otros trastornos mentales y encontrar manipulaciones ambientales que permitan potenciar la resiliencia de los animales. Desde un punto de vista traslacional es importante ampliar el estudio de la resiliencia como una pieza importante para desarrollar intervenciones que la mejoren y potencien, permitiendo a los individuos hacer frente al estrés social de una forma más eficaz, con el fin de prevenir el desarrollo de la adicción y otros trastornos mentales relacionados con el estrés.

Para alcanzar nuestros objetivos, primero caracterizamos el perfil conductual de los ratones adultos resilientes a los efectos a largo plazo de la DSRI sobre el CPL inducido por cocaína **(Estudio 1, ver Anexo).** Después, estudiamos la potenciación de los mecanismos de resiliencia mediante los efectos protectores del ejercicio físico voluntario **(Estudio 2, ver Anexo)**. A continuación, evaluamos la hipótesis de inoculación del estrés mediante

diferentes procedimientos como una breve separación maternal **(Estudio 3, ver Anexo),** la exposición a un estrés por inmovilización, la exposición a una sola derrota social o la visualización de una derrota social (DS vicaria) en la adolescencia **(Estudio 4, ver Anexo).** Por último, pretendimos confirmar si el perfil conductual de resiliencia descrito en el primer estudio también era aplicable a los animales resilientes expuestos a la DSRI durante la adolescencia temprana **(Estudio 5, ver Anexo).**

En el primer estudio, para caracterizar el perfil conductual de los ratones resilientes a los efectos de la DSRI, se utilizaron dos grupos de animales. El primero fue expuesto a un procedimiento de DSRI durante la adolescencia tardía, que consistió en cuatro encuentros agonísticos (separados por intervalos de 72 h, en los días postnatal (DPN) 47, 50, 53 y 56) con un ratón aislado (OF1), que resultó en la derrota del animal experimental (grupo DSRI). Cada episodio duró 25 minutos y constaba de tres fases, que comenzaban con la introducción del ratón experimental (intruso) en la jaula del oponente agresivo (residente) durante 10 minutos. Durante esta fase inicial, el intruso estaba protegido de los ataques por una rejilla metálica, que únicamente permitía la interacción a distancia y las amenazas del residente agresivo. A continuación, se retiraba la rejilla metálica de la jaula y tenía lugar la confrontación entre los dos ratones durante 5 minutos. En la tercera fase, se introducía la rejilla metálica de nuevo en la jaula para separar a los dos animales durante otros 10 minutos. De esta forma todos los ratones experimentales fueron derrotados por el oponente agresivo. El segundo grupo (control no estresado) se sometió al mismo protocolo, pero sin la presencia de un ratón "residente", por lo tanto, sólo realizaba la exploración de la jaula (grupo EXPL).

Entre las 24-48 horas después del último episodio de derrota (DPN 57 y 58), todos los animales realizaron una serie de pruebas conductuales: el laberinto elevado en cruz (LEC), que mide los niveles de ansiedad del animal; el *Hole-Board* que evalúa la búsqueda de novedad; y las pruebas de interacción social, suspensión de la cola y el *Splash Test*, que permiten detectar sintomatología depresiva *(Para más detalle véase el apartado 3. Material y Métodos)*. Tras un intervalo de 3 semanas, todos los ratones se sometieron al paradigma de CPL con una dosis subumbral de cocaína (1 mg/kg). En los DPN 77, 78 y 79 realizaron la fase de pre-condicionamiento (Pre-C). En este periodo los animales exploraron libremente los dos compartimentos que conforman el aparato y se registró el tiempo que pasaba en cada uno de ellos durante 15 minutos. Los animales que mostraban una fuerte aversión o preferencia incondicionada por un compartimento fueron excluidos del estudio. En los DPN 80, 81, 82 y 83 realizaron cuatro sesiones de condicionamiento (C1-C4) recibiendo 1 mg/kg de cocaína o solución salina antes de ser confinados durante 30 minutos en el compartimento asociado a la droga o de la solución salina, respectivamente. Finalmente, en la fase de post-condicionamiento (Post-C) en el DPN 84, los animales podían explorar de nuevo libremente los dos compartimentos durante 15 minutos. De esta forma evaluamos los efectos reforzantes condicionados de la cocaína, es decir, el valor positivo que habían adquirido las claves ambientales asociadas a esta droga.

Los resultados mostraron que la exposición a DSRI induce ansiedad ya que, en comparación a los controles, los ratones derrotados mostraron una reducción en todas las mediciones relacionadas con los brazos abiertos del LEC (excepto en la latencia). Además, la DSRI redujo la interacción social, la inmovilidad en la prueba de suspensión de la cola (TST) y el acicalamiento (*grooming*) en el *Splash Test*. La exposición a la DSRI también aumentó la

sensibilidad de los ratones a los efectos reforzantes condicionados de la cocaína, ya que sólo el grupo de animales derrotados mostró CPL tras el condicionamiento con 1 mg/kg de cocaína, una dosis que fue inefectiva en el grupo control no expuesto a estrés. Sin embargo, en el grupo de animales derrotados se distinguieron dos subgrupos, uno vulnerable a los efectos a largo plazo de la derrota sobre el refuerzo inducido por cocaína (que desarrolló CPL) y otro subgrupo resiliente que se comportó como el grupo control y por tanto no mostró CPL. Varios rasgos conductuales se relacionaron con la resiliencia al efecto potenciador de la DSRI sobre el CPL de cocaína. Los ratones resilientes mostraron menos sumisión durante los episodios de derrota, un menor porcentaje de tiempo en los brazos abiertos en el LEC, una baja búsqueda de novedad, una alta interacción social, una mayor inmovilidad en el TST y una mayor frecuencia de acicalamiento. Estos resultados sugieren que el perfil conductual de los ratones derrotados se caracteriza por una respuesta de afrontamiento activa durante los episodios de derrota (menos sumisión), una mayor preocupación por los peligros potenciales en entornos desconocidos (LEC *y hole-board*), una menor reactividad en una situación de estrés moderado (test de suspensión de la cola) y menos síntomas de tipo depresivo (*splash test*) tras el estrés. Por tanto, los resultados de este primer estudio ponen de manifiesto que varios rasgos individuales contribuyen a la resiliencia de un sujeto a las consecuencias negativas del estrés social (déficit de interacción social, anhedonia y mayor sensibilidad a las drogas). Desde un punto de vista traslacional nuestros resultados apoyan la observación del mundo real de que no todos los individuos expuestos al estrés social durante la adolescencia tardía padecen posteriormente trastornos mentales. Es importante identificar las características individuales que predicen la vulnerabilidad o resiliencia al

estrés para desarrollar estrategias dirigidas a incrementar la resiliencia de los sujetos más vulnerables a los efectos del estrés.

En el segundo estudio para evaluar si la exposición a ejercicio físico podía inducir una potenciación de los mecanismos de resiliencia, utilizamos cuatro grupos de animales. Dos grupos tenían acceso voluntario a ruedas de actividad durante la adolescencia temprana (DPN 21-47) durante una hora tres días por semana. Posteriormente, en los DPN 47, 50, 53 y 56, uno de estos grupos fue expuesto a estrés por DSRI (grupo ACT+DSRI) y el otro grupo sólo realizó exploración (grupo ACT+EXPL), siguiendo la misma metodología descrita anteriormente en el primer estudio. Otros dos grupos no fueron expuestos a actividad física y posteriormente (DPN 47, 50, 53 y 56) uno experimentó la DSRI (grupo CONTROL+DSRI) y el otro solo la exploración (grupo CONTROL+EXPL). Cabe señalar que el grupo en el que se expone al animal a un determinado ambiente o procedimiento (como la actividad física en este caso) sin posterior exposición a la DSRI es muy importante porque de este modo se evalúa si el procedimiento empleado afecta por sí mismo a las pruebas conductuales o al desarrollo del CPL inducido por cocaína. A continuación, los cuatro grupos realizaron la batería de pruebas conductuales descritas anteriormente en el primer estudio en el DPN 57-58 y a las tres semanas el CPL con 1 mg/kg de cocaína. Los resultados principales de este segundo estudio fueron que la exposición a ejercicio físico voluntario durante la adolescencia temprana evitó las consecuencias negativas del estrés social en el LEC, en el *splash test* y en el CPL de cocaína, ya que el grupo bajo condiciones de estrés que había realizado ejercicio físico (ACT+DSRI) no mostró sintomatología de ansiedad o depresión ni la potenciación de los efectos reforzantes condicionados de la cocaína observados en el grupo de animales derrotados sin ejercicio físico

previo (CONTROL+DSRI). Sin embargo, la exposición a actividad física no previene el déficit de interacción social provocado por la DSRI. Por ello, nuestros hallazgos apoyan la idea de que la actividad física promueve la resiliencia a algunos efectos negativos de un estrés social posterior y representa una excelente herramienta para la prevención del abuso de drogas u otros trastornos mentales relacionados con el estrés.

El fenómeno de la inoculación del estrés consiste en que la exposición de los sujetos a una situación de bajo nivel de estrés y a ser posible, controlable por el individuo, disminuye la respuesta desadaptativa a futuras exposiciones al estrés. En este sentido, nuestro tercer estudio consistió en separar a los ratones de la madre en edad temprana (DPN 9) por un corto período de tiempo (6 horas), mientras que los animales del grupo control se desarrollaron normalmente. Más tarde, siguiendo la metodología descrita en los estudios anteriores, evaluamos si este estrés agudo durante la infancia dotaba a los animales de una mayor resiliencia al estrés inducido por la derrota en la adolescencia tardía. Por tanto, se emplearon cuatro grupos de animales, dos expuestos a separación maternal (SM+DSRI y SM+EXPL) y dos controles sin separación maternal (CONTROL+DSRI y CONTROL+EXPL). Los resultados de este estudio mostraron que, independientemente de si la separación maternal había tenido lugar o no, se observó una reducción en las medidas de brazos abiertos en el LEC (excepto la latencia), de la búsqueda de novedad y de la interacción social en los ratones que experimentaron derrota (SM+DSRI y CONTROL+DSRI). Sin embargo, sólo se observó una mayor latencia de acicalamiento y una adquisición del CPL inducido por cocaína en los ratones expuestos únicamente a derrota (CONTROL+DSRI). Estos resultados indican que un episodio de SM previene algunos efectos de la exposición a la DSRI en la adolescencia tardía, como son la aparición de

sintomatología depresiva y la potenciación del CPL inducido por la cocaína. Sin embargo, la SM no modificó el comportamiento de evitación social y de ansiedad inducido por la DSRI. Por tanto, estos resultados sugieren que la inoculación contra el estrés en una etapa temprana de la vida mediante un breve episodio de SM aumenta la resiliencia posterior a algunos de los efectos negativos del estrés por DSRI, ya que evita el desarrollo de comportamientos depresivos en ratones derrotados a finales de la adolescencia y el aumento a largo plazo de su sensibilidad a los efectos reforzantes de la cocaína en la edad adulta.

En el cuarto estudio también evaluamos si otras manipulaciones ambientales durante la adolescencia temprana eran eficaces para incrementar la resiliencia. En el DPN 27 los animales no sufrieron ningún tipo de estrés (control) o fueron expuestos a uno de los siguientes protocolos para inducir inoculación de estrés: inmovilización durante 10 min (INM, estrés físico), una sola derrota social en un encuentro agonístico con un animal conspecífico agresivo (DS, estrés social) o una derrota social vicaria mediante la visualizaron la derrota social sufrida por otro animal de la misma cepa (DSV, estrés emocional). Posteriormente, en la adolescencia tardía (DPN 47, 50, 53 y 56) los animales de cada protocolo de estrés así como los controles fueron separados en dos grupos, un grupo fue expuesto a estrés social (DSRI) y el otro solo realizó la exploración de una caja vacía (EXPL). Por tanto, en este estudio se utilizaron ocho grupos de animales: CONTROL+DSRI, CONTROL+EXPL, INM+DSRI, INM+EXPL, DS+DSRI, DS+EXPL, DSV+DSRI y DSV+EXPL. Posteriormente todos los animales realizaron las pruebas conductuales descritas anteriormente (DPN 57-58) y a las tres semanas el CPL inducido por cocaína. Los resultados obtenidos en este estudio nos permiten confirmar la hipótesis de la inoculación al estrés

respecto a los efectos a largo plazo de la DSRI sobre las propiedades reforzantes de la cocaína. Mientras que los animales expuestos sólo a DSRI durante la adolescencia tardía (CONTROL+DSRI) adquieren un CPL inducido por una dosis de cocaína que no tiene efectos en los animales no estresados (CONTROL+EXPL), los animales derrotados en la adolescencia tardía que habían sido expuestos a cualquiera de los protocolos de inoculación de estrés en la adolescencia temprana (INM+DSRI, DS+DSRI y DSV+DSRI) no muestran esta mayor sensibilidad a los efectos reforzantes de la cocaína. Adicionalmente los protocolos de inoculación de estrés incrementan la resiliencia a otros efectos del estrés inducido por la DSRI aunque los resultados varían en función del protocolo utilizado. La exposición a inmovilización previene los efectos ansiogénicos de la DSRI en el LEC pero no los efectos pro-depresivos (disminución del grooming y evitación social) de la DSRI ni la reducción en la inmovilidad observada en el TST en animales expuestos a DSRI. Por su parte, la exposición a una sola derrota social también previene el efecto deteriorante de la DSRI sobre la interacción social mientras que la derrota social vicaria revierte efectos de la DSRI sobre la latencia de entrada en brazos abiertos del LEC.

Por otra parte, es importante señalar que alguno de los protocolos utilizados ejerce efectos negativos a largo plazo en ausencia de un estrés posterior. Por ejemplo, la inmovilización reduce la frecuencia de grooming en el splash test y la inmovilidad en el TST (estos efectos también son observado en animales expuestos a DS y DSV respectivamente) e incrementa la búsqueda de novedad (número de dips) y la sensibilidad de los animales a la cocaína, lo que podría indicar la aparición de sintomatología depresiva, un aumento en la reactividad de los animales al estrés y una mayor vulnerabilidad a los efectos reforzantes de la cocaína. Otros efectos parecen sugerir que la exposición al

estrés durante la adolescencia temprana podría inducir el desarrollo de un perfil de resiliencia, ya que la inmovilización incrementa la interacción social mientras que la DSV aumenta las entradas y el tiempo pasado en los brazos abiertos del LEC (lo que es considerado un indicador de menor ansiedad).

Por último, en el Estudio 5 investigamos la existencia de diferencias individuales en los efectos del estrés social durante la adolescencia temprana y estudiamos las características conductuales asociadas a la resiliencia. Para ello utilizamos el mismo diseño del primer estudio excepto que la DSRI o EXPL tuvieron lugar durante los DPN 27, 30, 33 y 36, las pruebas conductuales fueron realizadas los DPN 37-38 y el CPL tres semanas después (DPN 57-64). Los resultados de este estudio mostraron que los animales sometidos a DSRI en la adolescencia temprana, en comparación a los animales no estresados, mostraron un déficit de interacción social, una disminución en la frecuencia de acicalamiento en el *splash test* y un incremento en los efectos reforzantes de la cocaína. Sin embargo, la DSRI no produjo efectos significativos en el LEC, en el *hole-board* o en el test de suspensión de la cola. Además, al igual que en los animales expuestos a estrés durante la adolescencia tardía, podemos distinguir dos subgrupos dentro de los animales expuestos a DSRI en la adolescencia temprana, un subgrupo vulnerable y otro resiliente a los efectos del estrés sobre el CPL inducido por cocaína. Respecto a las características conductuales de los animales resilientes destacan una menor sumisión durante las derrotas, una ausencia de sintomatología depresiva (en el test de interacción social y en el *splash test)* y una mayor preocupación por los peligros potenciales (menor porcentaje de tiempo en los brazos abiertos del LEC).

Los estudios que componen la presente Tesis Doctoral han contribuido a incrementar el conocimiento sobre la resiliencia a los efectos del estrés social experimentado durante la adolescencia temprana o tardía. En primer lugar, es importante señalar que la DSRI produce efectos ligeramente diferentes en función de la edad de los animales en el momento de las derrotas. Tanto en la adolescencia temprana como en la tardía, la DSRI induce sintomatología depresiva a corto plazo (reduce la interacción social y el acicalamiento en el *splash test*) e incrementa los efectos reforzantes de la cocaína a largo plazo. Sin embargo, sólo en animales que experimentan la DSRI en la adolescencia tardía se observa sintomatología ansiosa en el LEC y una mayor reactividad ante un estresor ligero (reducción de la inmovilidad en el test de suspensión de la cola). Estos resultados sugieren que el estrés por DSRI tiene un mayor impacto cuando es experimentado durante la adolescencia tardía.

En segundo lugar, hemos caracterizado los rasgos conductuales que presentan los animales resilientes a los efectos de la DSRI en ambas etapas, especialmente respecto a la resiliencia a los efectos a largo plazo del estrés sobre las propiedades reforzantes de la cocaína. Tanto en animales expuestos al estrés durante la adolescencia temprana como tardía, una estrategia de afrontamiento activa durante las derrotas (baja sumisión) se asocia con una mayor resiliencia a los efectos a largo plazo de la DSRI, en concreto, los animales con baja sumisión no presentan una potenciación de los efectos reforzantes de la cocaína (que si se observa en los animales derrotados con mayores niveles de sumisión). Asimismo, independientemente de la etapa en que se experimenta el estrés social, los animales resilientes a los efectos de la DSRI sobre las propiedades reforzantes de la cocaína se caracterizan por ser resilientes a los efectos pro-depresivos de la derrota, es decir, no muestran un

déficit de interacción social ni una disminución del acicalamiento a corto plazo tras la derrota. Por el contrario, los animales derrotados vulnerables que muestran menores niveles de interacción social y acicalamiento que los animales no estresados muestran una mayor sensibilidad a los efectos de la cocaína y desarrollan CPL. Además, un menor porcentaje de tiempo en brazos abiertos del LEC predice resiliencia a los efectos del estrés sobre las propiedades reforzantes de la cocaína en los animales que experimentan la DSRI en ambas etapas de la adolescencia. Los estudios realizados también han puesto de manifiesto que algunos rasgos conductuales se asocian a la resiliencia o no en función de la etapa en que los animales experimentan el estrés social. Por ejemplo, el bajo nivel de búsqueda de novedad en el *hole-board* o un nivel de inmovilidad normal en el test de suspensión de la cola sólo predicen resiliencia en animales expuestos a la DSRI en la adolescencia tardía. Globalmente nuestros resultados indican que la edad es un factor importante a la hora de evaluar el impacto de la DSRI y las variables predictoras de resiliencia o vulnerabilidad a las consecuencias a corto y largo plazo del estrés social, aunque la estrategia de afrontamiento activa y la ausencia de sintomatología depresiva a corto plazo tras el estrés predicen resiliencia a sus efectos a largo plazo independientemente de la edad.

En tercer lugar, hemos demostrado que la resiliencia es un proceso dinámico que se puede desarrollar en función de las experiencias vitales experimentadas durante las etapas críticas del desarrollo. La exposición a una separación maternal breve en la vida temprana, la realización de actividad física durante la adolescencia o la exposición a estresores ligeros como una derrota social única, un estrés agudo por inmovilización o una derrota social vicaria pueden incrementar la resiliencia de los animales a los efectos de la exposición posterior a la DSRI durante la adolescencia tardía, especialmente

la inoculación de estrés previene la potenciación inducida por la DSRI de los efectos reforzantes de la cocaína.

El estudio de estrategias pro-resiliencia, aunque todavía se encuentra en una fase temprana de desarrollo, es crítico para diseñar futuras terapias ambientales y farmacológicas de prevención y tratamiento de los sujetos que desarrollan un TCS tras la exposición al estrés. Las investigaciones sobre la resiliencia a los efectos del estrés sobre las propiedades reforzantes de las drogas en modelos animales son prometedoras pero no están exentas de limitaciones, por ejemplo, la dificultad de determinar la intensidad y duración de la exposición a la adversidad, la definición de un criterio concreto para considerar que un animal es resiliente (ausencia o reducción del consumo de sustancias, resistencia a desarrollar un TCS o a la recaída en la búsqueda de drogas, etc.). Por otra parte, la incorporación de hembras y de animales en diferentes edades de desarrollo es crucial para comprender plenamente los diferentes aspectos de la resiliencia.

En conclusión, los resultados de nuestras investigaciones muestran que existen determinadas características neuroconductuales predictoras de resiliencia al estrés social y que es posible fomentar la resiliencia a los efectos negativos del estrés a corto y a largo plazo, tales como el desarrollo de trastornos de ansiedad, depresión o consumo de drogas. La caracterización de los animales resilientes es el primer paso para el desarrollo de estrategias conductuales y/o farmacológicas que puedan fomentar la aparición de una respuesta de resiliencia a los efectos negativos del estrés. Desde el punto de vista de la traslación, comprender cómo se desarrolla la resiliencia es de suma relevancia para el diseño de programas de entrenamiento que aumenten esta capacidad y promuevan mecanismos de afrontamiento, especialmente en los

sujetos más vulnerables al estrés. Además de reducir las conductas adictivas, el entrenamiento en resiliencia puede tener efectos positivos en salud mental, reduciendo la vulnerabilidad al desarrollo de trastornos de ansiedad, depresivos y cognitivos. Los avances en la identificación de los sustratos neurobiológicos de resiliencia ayudarán al desarrollo de intervenciones farmacológicas y psicológicas para mejorar la resiliencia ante la adversidad y el estrés.

ABSTRACT

The main focus of the present PhD Thesis was to study resilience to the development of a cocaine use disorder after exposure to stress. For this purpose, we used animal models of social stress (intermittent repeated social defeat, IRSD) and drug reward (paradigm of conditioned place preference, CPP), since we have previously seen that exposure to IRSD increases the rewarding effects of cocaine in the CPP paradigm in mice (García-Pardo et al., 2019; Calpe-López et al., 2020). Our aim was to identify animals that are resilient to the long-term potentiation of cocaine CPP induced by IRSD, to characterize the behavioral profile of such mice and to identify environmental manipulations that enhance their resilience. To characterize the profile of resilient mice several behavioral tests were performed shortly after IRSD, including the Elevated Plus Maze, Hole-Board, Social Interaction, Splash and Tail Suspension Tests.

In the first study we observed that exposure to IRSD in late adolescence induced negative consequences, such as anxiety- and depression-like symptoms (social interaction deficits and anhedonia), and an increased sensitivity to cocaine reward; however, mice that displayed an active coping strategy during defeat (low submission) were resilient to most of these effects. In the second and third studies we demonstrated that voluntary physical exercise during adolescence and a brief period of maternal separation at an early age enhanced resilience to the effects of IRSD in adulthood. In the fourth study, to assess the stress inoculation hypothesis, we demonstrated that a slight stressor in early adolescence, such as a brief period of immobilization, visualization of social defeat of another animal, or acute social defeat, protected against the negative consequences of subsequent exposure to IRSD.

In our last study, we explored the implication of age as a variable of resilience. In mice exposed to IRSD in early adolescence, low submission was associated with resilience to the depression-like effects and potentiation of cocaine CPP induced by IRSD.

From a translational point of view, our research may help the design of new preventive interventions and more effective treatments for substance use and other stress-related disorders, allowing individuals to cope with social stress in a more effective way. Characterization of the individual variables that confer resilience and identification of pro-resilience strategies, such as physical exercise, are critical for the development of behavioral, pharmacological and environmental therapies that can promote resilience in more vulnerable subjects.

PREFACE

The following papers compose this Doctoral Thesis.

→ **Calpe-López C.**, Martínez-Caballero M.A., García-Pardo M.P., Aguilar M.A. Resilience to the effects of social stress on vulnerability to developing drug addiction (2022). *World of Journal Psychiatry,* 12(1): 24-58. doi: 10.5498/wjp.v12.i1.24 PMID: 35111578. **(Review-Introduction)** (JCR 2021: 3.500, Q3)

- **Calpe-López, C**., Garcia-Pardo, M.P., Martinez-Caballero, M.A., Santos-Ortiz, A., Aguilar, M. A. Behavioral traits associated with resilience to the effects of repeated social defeat on cocaine-induced conditioned place preference in mice (2020). *Frontiers in Behavioral Neuroscience, 13*, 278. doi:10.3389/fnbeh.2019.00278.
  (Experimental Study 1) (JCR 2020: 3. 558, Q1)
- **Calpe-López, C**., Martínez-Caballero, M.A., García-Pardo, M.P., Aguilar, M.A. Intermittent voluntary wheel running promotes resilience to the negative consequences of repeated social defeat in mice (2022). *Physiology & Behavior*, *254*, 113916. doi: https://doi.org/10.1016/j.physbeh.2022.113916.
  (Experimental Study 2) (JCR 2021: 3.742, Q1)
- **Calpe-López C.**, Martínez-Caballero M.A., García-Pardo M.P., Aguilar M.A. Brief Maternal Separation Inoculates Against the Effects of Social Stress on Depression-Like Behavior and Cocaine Reward in Mice (2022). *Frontiers in Pharmacology*, 13:825522. doi: 10.3389/fphar.2022.825522.
  (Experimental Study 3) (JCR 2021: 5.988, Q1)

- **Calpe-López C.**, Martínez-Caballero M.A., García-Pardo M.P., Aguilar M.A. Inoculating against the Behavioral Effects of Intermittent Repeated Social Defeat in Adolescence Male Mice. (manuscript in preparation)
  (Experimental Study 4)

- **Calpe-López C.**, Martínez-Caballero M.A., García-Pardo M.P., Aguilar M.A. Resilience to the Short-and Long-term Behavioral Effects of Intermittent Repeated Social Defeat in Adolescent Male Mice. *Drug and Alcohol Dependence* (*under review*)
  (Experimental Study 5)

The following chapters of the book Methods for Preclinical Research in Addiction, Neuromethods, vol. 174, María A. Aguilar (ed.), https://doi.org/10.1007/978-1-0716-1748-9_8, © Springer Science+Business Media, LLC, part of Springer Nature 2022, are also included.

→ **Chapter 4: Claudia Calpe-López,** María Angeles Martínez-Caballero, María Pilar García-Pardo, María A. Aguilar. Modulation of the effects of alcohol, cannabinoids and psychostimulants by novelty-seeking trait.

→ **Chapter 8:** María Pilar García-Pardo, Jose Enrique De la Rubia-Ortí, **Claudia Calpe-López**, María Angeles Martínez-Caballero, María A. Aguilar. Influence of social defeat stress on the rewarding effects of drugs of abuse.

INDEX

ABBREVIATIONS

5-HT	Serotonin
ACTH	Adrenocorticotrophic Hormone
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
CORT	Glucocorticoids
CPP	Conditioned Place Preference
CRH	Corticotropin-Releasing Hormone
CUD	Cocaine Use Disorder
DA	Dopamine
DYN	Dynorphin
EPM	Elevated Plus Maze
HB	Hole-Board
HPA	Hypothalamic-Pituitary-Adrenal axis
IRSD	Intermittent Repeated Social Defeat
KOR	Kappa-Opioid Receptor
LC	Locus Coeruleus
MS	Maternal Separation
NAcc	Nucleus Accumbens
NA	Noradrenaline
PFC	Prefrontal Cortex
PND	Post Natal Day
PTSD	Post-Traumatic Stress Disorder
SD	Social Defeat
SI	Social Interaction

SNS	Sympathetic Nervous System
SUD(s)	Substance Use Disorder(s)
TST	Tail Suspension Test
UCS	Unpredictable Chronic Stress
VSDS	Vicarious Social Defeat Stress
VTA	Ventral Tegmental Area
VWR	Voluntary Wheel Running

1. INTRODUCTION

1.1 General Introduction

Drug addiction affects multiple motivational mechanisms and can be conceptualized as a mental disorder that progresses from positive reinforcement (binge/intoxication stage) to negative reinforcement (withdrawal/negative affect stage) (Koob et al., 2014). Thus, substance use disorders (SUD) may emerge from an individual's attempt to limit negative affective states and symptoms linked to stress. Indeed, SUD is highly comorbid with chronic stress, traumatic stress, or post-traumatic stress disorder (PTSD), but treatments approved for each pathology individually often fail to have a therapeutic efficiency in comorbid patients (Leconte et al., 2022). Currently, SUDs are highly prevalent worldwide (Connery et al., 2020). They involve vicious cycles of binges followed by intermittent periods of abstinence with repeated relapses, despite treatment and adverse medical and psychosocial consequences. There is compelling evidence that early and adult stressful life events are risk factors for the development of addiction and serve as triggers for relapses. However, the fact that not all individuals dealing with traumatic events develop drug dependence suggests the existence of individual and/or familial factors of resilience that provide protection in these mentally healthy persons (Cadet, 2016).

Resilience is a relatively new and unclear concept, although it is increasingly used in everyday conversations and across disciplines. It is defined as a protective factor that makes individuals more resistant to negative events, thus leading to positive developmental outcomes (Stainton et al., 2019). Resilience is a positive adaptation to stressful situations and represents a mechanism to face and overcome difficult experiences; in other

words, it is an individual's ability to adapt to change, resist the negative impact of stress and avoid dysfunction. In summary, it represents the ability to return to what was once a "normal" or healthy state after trauma, accident, tragedy or illness. Resilience experts believe that resilience contribute to the advancement of health and, if sick, to alleviate illness, accelerate and create healing conditions (Babic et al., 2020).

Research has consistently found that a favorable exchange with one's proximal social environment has positive effects on both mental health and wellbeing (Tough et al., 2017). Nevertheless, one of the main sources of stress is social interaction (Sánchez-Salvador et al., 2021; Miller, R., & Kirschbaum, C., 2019); namely, problems or friction that arise in relations with colleagues and/or superiors, neighbors, friends or family. Social relationships in humans and animals are governed by rules of social organization that modulate inhibitory control and coping strategies against stress (Sánchez-Salvador et al., 2021).

In short, good social support helps us to cope with problems and protects us from adversity, but social interaction can itself cause stress. In this way, social relationships can be both a protective factor and a risk factor for the development of mental illnesses, including SUD. The way in which the organism copes with daily stressors varies among individuals, is multifactorial, and changes throughout an individual's life. The fact that not everyone develops a mental illness due to chronic stress is due to resilience. Therefore, our main objective of this Doctoral Thesis is to characterise animals that are resilient to the behavioral effects of social stress and the potentiation of the rewarding properties of cocaine induced by stress. In addition, we will evaluate the behavioral profile associated with resilience in function of the developmental stage of the animals at which they are exposed

to social stress. Finally, we will attempt to enhance this resilience through environmental manipulations.

The present thesis is composed of five experimental studies, three of which have been published, the forth is under review for publication and the fifth is in preparation. The theoretical framework is based on a review article in which the author of this thesis is the first author. For a better understanding of the methodology used, two chapters of a book on Neuromethods in which the author of this thesis has participated are included in the annex. The two main animal models used in all the studies were the *Conditioned Place Preference (CPP) and the Intermittent Repeated Social Defeat (IRSD)* paradigms, which are designed to study the rewarding properties of drugs of abuse and to induce social stress, respectively. Moreover, all the studies performed as part of this thesis employed several behavioral tests, including the Elevated Plus Maze, Hole-Board, Social Interaction, Splash and Tail Suspension Tests.

The results of the first study help us to characterize the resilient profile of male mice in the face of social defeat. The data show that several individual traits contribute to a subject's resilience to the negative consequences of social stress (social interaction deficit, anhedonia and increased sensitivity to drugs). The second and third studies show how voluntary physical exercise during adolescence and a brief maternal separation at an early age can enhance resilience to stress in adulthood. In the same vein, we tested the stress inoculation hypothesis in our fourth study, our results demonstrating how a minor stressor, such as immobilization, visualization of social defeat and acute social defeat, in early adolescence protects against future stressors and their negative consequences. In our final experiment, we have explored the

implication of age as a variable of resilience (early adolescence and adulthood). We trust that our research will contribute to a better understanding of SUDs and to the development of more effective preventive programs or treatments for those affected by enhancing stress resilience.

1.2 About Cocaine

Cocaine, like other substances, such as caffeine, nicotine, or morphine, is a plant product. Specifically, it is an alkaloid found in the leaves of a shrub called *Erythroxylon coca*, endogenous of South America, Mexico, Indonesia, and the West Indies (Goldstein et al, 2009). It is known that, as long ago as 3000 BC, the natives of these areas chewed this leaf for its anti-fatigue, appetite-reducing and energy-enhancing properties, as well as using it in numerous rituals. The German chemist Friedrich Gaedecke was the first to isolate the alkaloid from the leaf in 1855 (Van Dyke & Byck, 1982). However, Albert Niemann was the first to describe the drug and name it (Kleber & Gawin, 1986). In 1880, cocaine began to be used for eye, nose, and throat surgery due to its anesthetic properties and as a constrictor of blood vessels, as it limited bleeding. Coca leaves have been used in teas and wines (*Vin Mariani*) and incorporated into beverages such as Coca Cola™. From that moment on, its consumption increased in line with reports of heart attacks, spontaneous death and the development of SUD.

Nowadays, there are a large number of synonyms and street names for cocaine: *Coca, Coke, Snow, Blow, Base, Crack, Nose Candy, Pimp´s drug, Caviar, Yeyo, Dama Blanca.* Its chemical name is *Benzoyl-methyl-ecgonine* and the molecular formula is $C_{17}H_{21}NO_4$. The biochemical structure of

cocaine consists of three parts: a lipophilic group, a hydrophilic group and an aliphatic group *(See **Fig.1**)*.

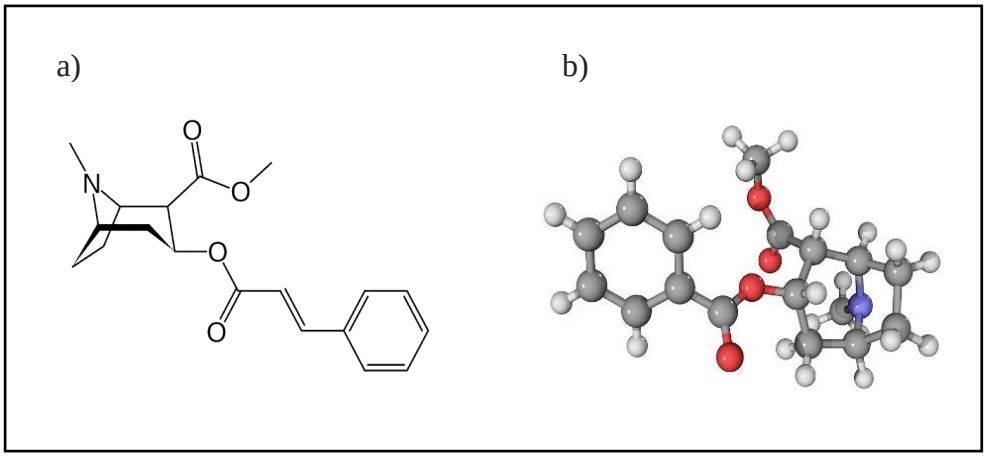

Fig.1. The biochemical structure of Cocaine. *(a) 2D-Structure and (b) 3D-Structure (design by Laguna).*

1.2.1 Presentation

Cocaine is available in two forms on the street: Hydrochloride salt (a powder) that can be administered intranasally (i.e. snorted) or dissolved in water and injected, and the "base" forms, which include all forms that are not neutralized by acids to form hydrochloride salt. The base forms are known as *Freebase* or *Crack*, depending on the manufacturing method.

Freebase is made by dissolving cocaine hydrochloride in water, adding a base such as ammonia, and then adding a solvent, usually ether. The cocaine base is dissolved by the ether and extracted by evaporation. There is a chance that the highly volatile ether remains in the mixture and can cause burns (Khalsa et al., 1992). On the other hand, *Crack* is produced by dissolving cocaine hydrochloride in water, mixing it with ammonia or sodium bicarbonate

(baking soda), and heating this mixture to remove hydrochloride (Brownlow and Pappachan, 2002). The remaining product is a soft mass that becomes hard when it dries. The name "crack" derives from the crackling sound produced when the mixture is smoked (Kowalchuk and Reed, 2016). In its base form, cocaine can be smoked because it melts at a much lower temperature (80°C) than cocaine hydrochloride (180°C). With the increased prevalence of crack, made possible by a simpler and less dangerous process of consumption, the use of *Freebase* has declined. Although crack is typically smoked, some users dissolve it with lemon juice and inject it (Weiss, 2020).

1.2.2 Epidemiology

Psychomotor stimulants, such as cocaine, are the most commonly used prohibited substances after cannabis. Their use has reached epidemiological proportions worldwide and is one of the most common causes of death in many countries. In the European Union, surveys indicate that nearly 2.2 million 15- to 34-year-olds (2.1 % of this age group) used cocaine in 2020 (European Drug Report, 2021).

Cocaine remains the second most commonly used illicit drug in Europe, and consumer demand makes it a lucrative part of Europe's drug trade and criminal activity. The record 213 tonnes of the drug seized in 2019 indicates an expanding supply in the European Union. Cocaine purity has been increasing over the last decade, and the numbers of people initiating treatment for the first time have risen over the last 5 years (European Drug Report, 2021).

The use of cocaine has negative effects on the cardiovascular system and is one of the causes of serious cardiovascular pathologies ranging from

abnormal heart rhythms to heart attacks and sudden cardiac death. Reactive oxygen species generation, formation of toxic metabolites, and oxidative stress play a significant role in cocaine-induced cardiotoxicity (Georgieva et al., 2021). These and other indicators signal an increased likelihood of cocaine-related health problems.

1.2.3 Metabolism

Smoking and injecting cocaine are the fastest routes to the cerebral circulation, taking only seconds to feel the effects. These pathways are also the most addictive to humans (Warner, 1993; Shanti and Lucas, 2003). On the other hand, when cocaine is snorted, a feeling of euphoria occurs one to five minutes later. Smoking and injecting cocaine produces a rush and then a high, whereas snorting cocaine produces only a high (Egred and Davis, 2005). This is due in part to the fact that the amount of cocaine absorbed in the nasal mucosa has 20-60% bioavailability, while the bioavailability of smoked cocaine is about 70% (Cone, 1998). In addition, inhaled cocaine causes local vasoconstriction, thereby inhibiting faster absorption (National Center for Biotechnology Information, 2022).

Benzoylecgonine and *ecgonine methyl ester* are the two inactive metabolites that account for more than 80% of the known metabolites of cocaine. Less than 10% of cocaine is N-demethylated by the liver into a toxic metabolite called *norcocaine* (Shimomura, Jackson, & Paul, 2019). Either way, cocaine and its metabolites are detected in the urine three to six hours after ingestion (Shanti and Lucas, 2003). With a half-life of approximately one hour, less than 5% of cocaine appears unchanged in the urine; therefore, drug tests are

designed to detect *benzoylecgonine*, as its concentration in urine is 50 to 100 times higher than cocaine (Shimomura et al., 2019).

1.2.4 Neurobiology

Cocaine's main mechanism of action is to block the reuptake of dopamine (DA) from presynaptic receptors in the Central Nervous System (CNS) *(See **Fig. 2***). The drug also attaches to the transporter and blocks the normal recycling of other neurotransmitters, such as Noradrenaline (NA) and Serotonin (5-HT), at the synaptic junction. This entails a build-up of monoamines that induce a constant stream of biochemical stimulation in the synaptic cleft, increasing the feeling of pleasure felt by cocaine users. The main effects of cocaine include feelings of euphoria – a result of the excess of DA - increased energy due to the increase of NA, and enhanced confidence concomitant with the enhancement of 5-HT (Nestler, 2005). Another effect, used in medicine, is local anesthesia. Cocaine shuts off the conduction of sensory impulses by reacting with the neuron membrane to block ion channels. Due to this blockage, the ion exchange that is normally responsible for electrical signals cannot propagate along the axon, and sensory messages are not received by the CNS, thereby exerting anesthetic properties (Liu et al., 2014). The neural system most affected by cocaine is the mesolimbic DA system, and mainly the Ventral Tegmental Area (VTA) and the Nucleus Accumbens (NAcc), which are fundamental to the positive reinforcement of addiction and constitute the regions that regulate pleasure and motivation (Koob et al., 1998; Volkow et al., 2003; Nestler, 2005; National Center for Biotechnology Information, 2022).

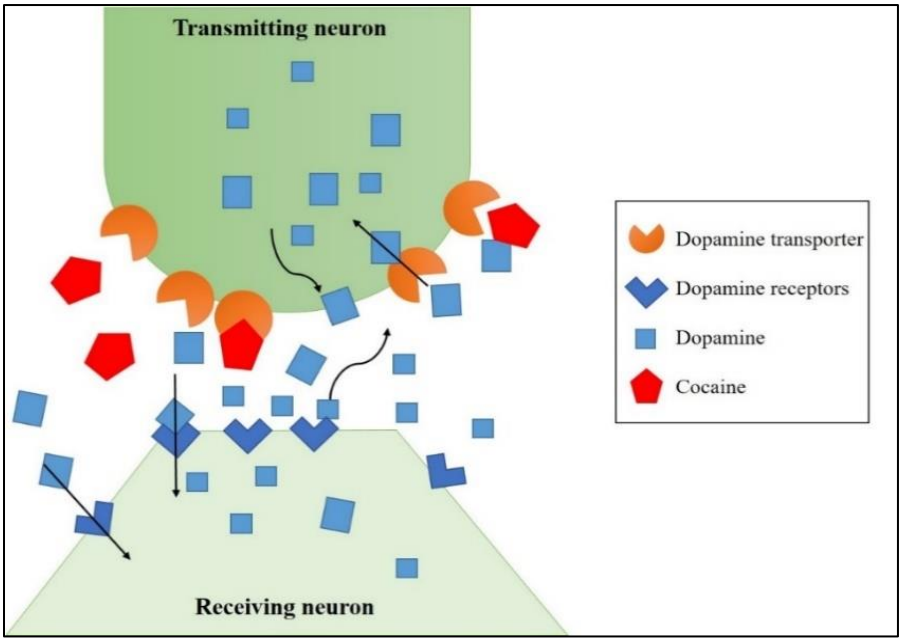

Fig.2. Cocaine in the synapsis.

Cocaine Use Disorder is a serious problem, and there is no consensus regarding optimal treatment strategies. Therefore, the present thesis seeks to explore this subject in more depth in order to provide more resources for the prevention of addiction.

1.3 Stress and Cocaine Use Disorder

In the following sections, we first define the concept of stress and its influence on the development of physical and mental illnesses. We then explain the physiological response to stress and the relationship with SUD. Finally, we focus on the animal models used to study the effects of social stress on the rewarding properties of cocaine.

1.3.1 Stress and Mental Disorders.

Stress is a physiological reaction to environmental conditions, and each person copes with it in a different way. Social interaction is one of the main sources of stress; namely, problems or quarrels which arise among colleagues and/or superiors, among friends and family or within the home. For this reason, social stress plays an important role in the incidence of several mental diseases, including the development of addictive behaviors (Atrooz et al., 2019; Koo and Wohleb, 2021; Cattaneo and Riva, 2015).

Short-term stress can help to achieve goals and most likely provides us the pleasant eustress (from the Greek eu meaning good, as in euphoria) involved in achieving fulfillment and victory, thereby avoiding the self-destructive distress of frustration and failure (Selye, 1975). However, when stress becomes chronic and hormones of stress are prolonged over time, damaging symptoms appear in the organism (Satsangi and Brugnoli, 2018; Sarjan and Yajurvedi, 2018). McEwen coined the term “allostatic load” to describe the burden of life’s experiences which accumulates and affects the body and the brain as an individual is exposed to repeated stress (McEwen and Stellar, 1993). It is well known that chronic stress has an extremely negative impact

on health and social life. Physical ailments such as joint pain, back pain, neck pain, headache, heartburn, diarrhea may appear, as well as the development of other pathologies such as irritable bowel syndrome, skin reactions, fatigue, hypertension, breathing difficulties and dizziness (Bennett et al., 1998; Konturek et al., 2011; Liu et al., 2017). In addition to these consequences for physical health, there is extensive literature to demonstrate the link between chronic stress, cognitive function and mental health (see the review Marin et al. 2011). In this way, the prefrontal cortex (PFC) is particularly sensitive to stress exposure, and deficits in its structure and function are common in mental illness (Hains et al., 2015). Exposure to acute stress causes the PFC to rapidly "switch off" through a cascade of intracellular signaling events, while repeated exposure to stress leads to additional architectural changes (Arnsten, 2009). In particular, enhanced stress-induced catecholamine release in the PFC activates DA D1 and alpha-1 and beta noradrenergic receptors, which activates cAMP-calcium signaling in spines and opens nearby K+ channels, thus weakening synaptic connections (Hains et al. 2015). Over time, this sequence of events reduces the activation of PFC neurons and impairs working memory in rodents (Murphy et al., 1996) and monkeys (Arnsten and Goldman-Rakic, 1998). Similar architectural changes are observed in human PFC (Qin et al., 2009), where brain imaging studies show that repeated stress is associated with decreased PFC gray matter (Ansell et al., 2012) and lower connections (Liston et al., 2009). Indeed, chronic exposure to uncontrollable stress decreases the number of spines and dendrites in the PFC (Woo et al., 2021). Stress also affects gene expression and induces dendritic remodeling in the hippocampus (McEwen and Magarinos, 1997; Marrocco et al., 2017), the most important brain area for explicit memory.

As we have commented on before, chronic stress is also considered one of the main drivers of the vicious cycle of SUD, which plays an important role during acquisition and maintenance of drug abuse and relapse to consumption after a period of withdrawal. SUD can arise from an individual's attempt to limit negative affective states and stress-related symptoms experienced during short-term or protracted abstinence. In fact, SUD is highly comorbid and prevalent with chronic stress, traumatic stress, or PTSD (Saunders et al., 2015; Tiet and Moos, 2021; Leconte, 2022).

1.3.2 Physiological Response to Stress and Substance Use Disorder.

When stressful physical or mental stimuli are present, the body prepares to combat or avoid them by releasing a cascade of hormones. Physiological activation of the hypothalamic-pituitary-adrenal (HPA) axis is the main control system for stress reactions and regulates many other bodily processes such as digestion, the immune system, sexuality, mood and emotions (Petrescu et al., 2018; Vegiopoulos and Herzig, 2007; Toufexis et al., 2014; Schauenstein et al., 2000). The paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH), which leads to the release of adrenocorticotropic hormone (ACTH) from the adenohypophysis, thus stimulating the production of glucocorticoids (corticosterone in rodents and cortisol in humans) by the cortex of the adrenal glands. Negative feedback mechanisms also exist in the HPA axis; for example, glucocorticoids block the production of CRH and ACTH *(See Fig.3).* Besides regulating the adrenal stress response, CRH is a neuropeptide that is widely distributed in the brain, mainly in the circuitry of extended amygdala, which includes the central nucleus of the amygdala, the bed

nucleus of the stria terminalis, and the shell of the NAcc (Koob, Brain Res, 2009). There is a cross-talk between extrahypothalamic CRH and stress-altered DA release, with an increase in the PFC and a decrease in the NAcc (Koob et al., 2014). Moreover, stress activates the sympathetic nervous system (SNS), which induces adrenomedullary release of NA. Stress also stimulates the noradrenergic system of the brain, causing NA release from the locus coeruleus (LC) to the amygdala, hippocampus, hypothalamus, and PFC (Aston-Jones and Cohen, 2005; Strawn and Geracioti, 2008).

Elevated glucocorticoids can cause damage and atrophy of neurons in different areas of the brain involved in memory and emotional behavior, such as the hippocampus and amygdala, inducing physical and psychological problems. In addition, chronic stress interferes with the action of neurotrophic factors responsible for the formation and strengthening of new neurons and synaptic connections, especially in the hippocampus, such as brain-derived neural factor (BDNF). For example, it has been noted that the volume of this structure and reduced levels of BDNF in subjects exposed to prolonged stress may be a risk factor for the development of PTSD (Duman, 2009; Tural et al., 2018; Kozlovsky et al., 2007).

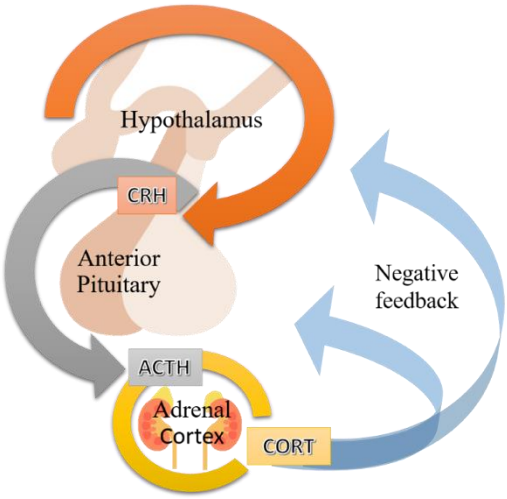

Fig.3. Schematic hypothalamic-pituitary-adrenal (HPA) Axis. *The HPA axis is central to homeostasis, stress responses, energy metabolism, and neuropsychiatric function. CRH (corticotropin-releasing hormone), ACTH (adrenocorticotropic hormone) and CORT (glucocorticoids: cortisol in humans and corticosterone in rodents).*

The influence of stress on vulnerability to developing addictive-like behaviors is mediated by the dysfunction of DA systems induced by stress. Excessive drug intake activates CRH in the extended amygdala, leading to anxiety-like states, and in the medial PFC, inducing deficits in executive function that can facilitate the transition to compulsive drug consumption (Koob et al., 2014).

Researchers have shown that there is a complex relation between HPA axis activation and the neurobehavioral and hormonal effects of cocaine. This

substance stimulates a number of neurochemical and hormonal structures, which are also activated through exposure to stress (Piazza and Le Moal, 1998; Koob, 1999; Goeders, 2002). Cocaine administration increases plasma levels of ACTH, β-endorphin and corticosterone. These cocaine-induced increases in adrenocorticosteroids seem to be mediated by the cocaine-induced release of CRH from parvocellular neurons in the paraventricular nucleus (Sarnyai et al., 2001; Goeders, 2002). In addition, cocaine can also affect CRH activity in areas located outside the hypothalamus, since acute cocaine decreases CRH-like immunoreactivity in the hypothalamus, hippocampus, and frontal cortex, while cocaine increases it in the amygdala (Goeders, 2002). In clinical studies, the acute intravenous administration of cocaine increases the secretion of cortisol and ACTH in chronic cocaine users, as does smoked cocaine (Mello and Mendelson, 1997). The intranasal administration of cocaine has also been shown to increase cortisol secretion in male volunteers without a history of drug abuse. Plasma cortisol, β-endorphin, and ACTH are elevated in cocaine addicts on the day of admission into treatment centers, and cocaine-dependent individuals often display abnormal patterns of HPA axis activity (Mello and Mendelson, 1997).

The HPA axis is involved in the self-administration of psychostimulant drugs (Goeders, 1997; Moffett and Goeders, 2005; Gómez-Román et al., 2016; Hofford et al., 2018). Stress and cocaine interact to affect reward differently during the various phases of cocaine self-administration and withdrawal (Goeders, 2002). The acquisition of amphetamine and cocaine self-administration is enhanced in rats exposed to a wide variety of either physical (e.g., social isolation or tail pinch) or social stress (e.g., exposure to the threat of an attack from an aggressive male rat). During the acquisition phase of drug use, corticosterone seems to be crucial, since self-administration does

not occur unless levels of this stress hormone rise above a critical threshold. Increased circulating corticosterone levels also increase sensitivity to low doses of cocaine, possibly stemming from a DA-related sensitization phenomenon, suggesting that stress exposure increases the susceptibility of individuals to cocaine-induced brain injury. In this context, drugs affecting the synthesis and/or secretion of corticosterone decrease ongoing, low-dose cocaine self-administration (Goeders, 2002; Goeders, 2003).

CRH seems to play a more prominent role in the maintenance of cocaine self-administration and may even be involved in incentive motivation for the drug. During the abstinence phase, exposure to stressors or cocaine-associated cues can stimulate the HPA axis to remind the individual of the positive effects of cocaine, thus producing craving and promoting relapse. Cocaine can induce anxiety and panic in humans and anxiogenic-like responses in animals through its effects on CRH release (Goeders, 1997; Goeders, 2002). Dysregulation of the HPA axis has been associated with craving and early relapse among individuals with SUD (Ligabue et al., 2020), and CRH and corticosterone are critical for the stress- and cue-induced reinstatement of extinguished cocaine-seeking behavior in rodents (Hadad et al., 2016; Bernardi et al., 2017).

In addition, the function of the kappa-opioid receptor (KOR) and its endogenous ligand, dynorphin (DYN), is increased during traumatic stress or drug abuse (Laconte et al., 2022). The DYN/KOR system is cross-regulated with CRH in the brain and DYN is a potent negative modulator of DA signaling in reward and fear circuits. The DYN/KOR system is involved in negative reinforcement once the euphoric effects of a drug of abuse end, and chronic drug use induces DYN/KOR activation, which facilitates tolerance,

dependence and relapse after withdrawal (Koob et al., 2014). The development of drugs that reduce HPA axis activity, especially in response to cocaine-associated cues, represent an exciting avenue for the discovery of novel pharmacotherapies for the treatment of cocaine addiction in humans (Goeders, 2002b). In this way, KOR antagonists could be a new target to treat SUD and PTSD comorbidity (Laconte et al., 2022). Continued investigations into how stress and the subsequent activation of the HPA axis affect cocaine self-administration will no doubt result in the identification of more effective and efficient treatments for cocaine use disorder (CUD) in humans. Stress reduction, either alone or in combination with pharmacotherapies that target the HPA axis, may prove beneficial in reducing craving and promoting abstinence in individuals seeking treatment for cocaine addiction.

1.3.3 Effects of stress on cocaine reward in animal models.

Animal models have allowed the study of the relationship between stress and addictive disorders.

There are multiple techniques to induce stress in experimental rodents. Some of them use pharmacological stressors, such as daily administration of corticosterone (Brachman et al., 2016). For example, Mc Reynolds et al. (2017) used a conditioned place preference/reinstatement paradigm in mice to directly test the hypothesis that corticosterone potentiates cocaine-primed reinstatement. Other paradigms use physical stressors, such as footshock (Erb et al., 2004; Brachman et al., 2016; Berton et al., 2007; Fleshner et al., 2011) or immobilization (Ono et al., 2012). In the paradigm of learned helplessness animals are exposed to inescapable, unpredictable and uncontrollable foot-

shocks (Erb et al., 2004; Brachman et al., 2016; Berton et al., 2007; Fleshner et al., 2011). After such exposure to stress, a subset of susceptible animals develops coping deficits to deal with the unavoidable shocks (learned helplessness), while another subset of resilient animals displays escape responses similar to non-stressed animals (Berton et al., 2007). Pretreatment with a daily 20 min footshock stress for 5 days was shown to enhance the cocaine-induced increase in extracellular DA levels in shock- versus sham shock-pretreated rats (Sorg and Kalivas, 1991). Another study in footshock-stressed rats demonstrated that cocaine induced a reduction of anxiety-like behavior, an aggravation of recognition memory decline, and an impairment of extinction memory (Lguensat et al., 2021).

Several studies have proved that restraint stress increases cocaine conditioned place preference (CPP) in mice (Tung et al., 2016; Chu et al., 2020; Wada et al., 2020). In the present thesis, we have employed immobilization stress, by which mice are acutely restrained for 15 min (the animal is gently introduced in a cylindrical glass tube in which is impossible for it to turn) *(see section 3. Material and Methods)*, as our objective was to expose the animals to a slight stress.

Another frequently used model is unpredictable chronic stress (UCS), also called chronic mild stress, which is based on a combination of physical and psychosocial stressors (Delgado y Palacios et al., 2011). After exposure to this model, most animals (about 70%) show anhedonia–like symptoms (less sucrose consumption), reduction of hippocampal volume, and alterations in glutamate metabolism, although there is a subset of resilient animals that do not exhibit these changes (Delgado y Palacios et al., 2011). Exposure to UCS enhances the acquisition of cocaine CPP in cannabinoid CB1 KO mice, but

does not significantly alter the effects of cocaine in WT mice, suggesting a role for the CB1 receptor in the response to stress, as well as in the effects of cocaine (Miller et al., 2008). Another study has shown that UCS, but not chronic predictable stress, increases the locomotor activating effects of cocaine (that correlated positively with corticosterone levels) and enhanced the place conditioning effects of this drug, increased CPP with a low dose and induced place aversion with a high dose of cocaine (Haile at el., 2001).

The model of chronic social defeat stress (CSDS) is the most used animal model to study the effects of stress and has more ethological and ecological validity than others. As we have explained before, one of the most frequent types of stress faced by humans is the chronic social stress derived from problems with social interaction (family or friend relationships, work-place stress, bullying, etc.). In the CSDS model, brief episodes of aggression from a more aggressive conspecific in the resident-intruder paradigm result in the defeat of the experimental animal (intruder), which usually shows anxiety- and depression-like symptoms (Vannan et al., 2018; Bartolomucci et al., 2009; Nestler and Hyman, 2010; Hollis and Kabbaj, 2014; Czéh et al., 2016). In the most widely employed SD model, rats or mice are exposed to SD for 10 days. Each day, the experimental animal undergoes 10 min of physical attack by the aggressive opponent, followed by 24 h of sensory contact. Subsequent addiction-like behaviors depend on the intensity, duration, frequency, and intermittency of the confrontation episodes, but CSDS exposure induces an escalation of cocaine and alcohol consumption (Shimamoto, 2019). In addition, all mice exhibit heightened reactivity of the HPA axis, deficits in exploration (interpreted as increased anxiety) and polydipsia (Krishnan et al., 2007).

A variation of the classic 10-day CSDS paradigm consists of exposing animals to intermittent repeated SD (IRSD); usually, four episodes of defeat separated by intervals of 72 h. The IRSD model is frequently employed in studies about the influence of social stress on vulnerability to developing drug addiction. Exposure to IRSD has also been shown to increase the rewarding effects of drugs of abuse (Aguilar et al., 2013; Ellenbroek et al., 2005; Burke et al., 2011; Newman et al., 2018). In our laboratory, mice exposed to IRSD during early or late adolescence exhibit a long-term enhanced sensitivity to the rewarding effects of drugs of abuse such as MDMA (García-Pardo et al., 2015), alcohol (García-Pardo et al., 2016; Rodriguez-Arias et al., 2016) or cocaine (Covington et al., 2001; Aguilar et al., 2013; Calpe-López et al., 2021). We have used this procedure in all the experiments included in this Thesis *(for a more detailed description see section 3-Material and Methods).*

Several types of moderate stressors have been used to study the phenomenon of "stress inoculation". According to this hypothesis, exposure to a mild stress in early life or adolescence increases the resilience to a subsequent stressful experience later in life with respect to several physiological and behavioural parameters (Ashokan et al., 2016; Hsiao et al., 2016; Qin et al., 2019). For example, infant rats exposed to intermittent foot shocks subsequently respond more effectively in novel situations than non-stressed rats (Levine, 1962). In addition, the combination of maternal deprivation during early life with UCS during adolescence promotes greater resilience in adulthood than maternal deprivation alone (Ricon et al., 2012). In the present thesis, we have used a brief episode of maternal separation early in life and several acute stressful events in adolescence (an episode of immobilization, social defeat or vicarious social defeat) in order to induce stress inoculation (*see Section 3. Material and Methods*). During chronic exposure to stress, behavioral

strategies that limit the experience of stress may promote resilience. During chronic SD, animals that engage in less submissive postures when threatened and attacked by the opponent show less social avoidance, suggesting that this behavioral coping strategy reduces the effects of the stress (Wood et al., 2010). Behavioral manipulations have also been used to reduce the effects of stress and increase resilience; for example, exposure to environmental enrichment (Hutchinson et al., 2012) or physical exercise (Holmes, 2014; Sciolino et al., 2015). We have also evaluated the effects of physical exercise on resilience to the effects of IRSD (*see Section 3. Material and Methods*).

In conclusion, available evidence supports the role of stress in enhancing susceptibility to progressing from drug abuse to a SUD. Defeat experiences in animals have been proven to be a risk factor in all the stages of drug addiction. Social stress can enhance the psychomotor response and the unconditioned and conditioned rewarding effects of cocaine. Moreover, SD stress can act as a precipitant factor in the reinstatement of drug seeking in the self-administration and CPP paradigms.

A more in-depth description of this topic can be found in Chapter 8: "Influence of Social Defeat Stress on the Rewarding Effects of Drugs of Abuse" of the book "Methods for Preclinical Research in Addiction", Neuromethods 174, Humana Press, in which the author of the present thesis has participated *(See ANNEX).*

1.4 Resilience to Stress and Substance Use Disorder

I have reviewed this issue in my paper "*Resilience to the effects of social stress on vulnerability to developing drug addiction"* **Claudia Calpe-López**, Maria A Martínez-Caballero, Maria P García-Pardo, Maria A Aguilar, recently published in World Journal of Psychiatry 2022; 12(1): 24-58 [PMID: 35111578 DOI: 10.5498/wjp.v12.i1.24].

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REVIEW

Resilience to the effects of social stress on vulnerability to developing drug addiction

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Abstract

We review the still scarce but growing literature on resilience to the effects of social stress on the rewarding properties of drugs of abuse. We define the concept of resilience and how it is applied to the field of drug addiction research. We also describe the internal and external protective factors associated with resilience, such as individual behavioral traits and social support. We then explain the physiological response to stress and how it is modulated by resilience factors. In the subsequent section, we describe the animal models commonly used in the study of resilience to social stress, and we focus on the effects of chronic social defeat (SD), a kind of stress induced by repeated experience of defeat in an agonistic encounter, on different animal behaviors (depression- and anxiety-like behavior, cognitive impairment and addiction-like symptoms). We then summarize the current knowledge on the neurobiological substrates of resilience derived from studies of resilience to the effects of chronic SD stress on depression- and anxiety-related behaviors in rodents. Finally, we focus on the limited studies carried out to explore resilience to the effects of SD stress on the rewarding properties of drugs of abuse, describing the current state of knowledge and suggesting future research directions.

Key Words: Resilience; Stress; Depression; Drug addiction; Animal models; Social defeat

Core Tip: Preclinical research on drug addiction has focused on the factors that enhance vulnerability to develop drug addiction. Recent studies of resilience have determined

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the neurobehavioral traits that confer protection against developing an addictive disorder after stress exposure. Active coping strategies to face the stressor and the absence of depression-like symptoms are consistently associated with resilience to the stress-induced potentiation of the rewarding effects of cocaine and alcohol. Unravelling the neurobiological substrates of resilience is key to developing pharmacological and psychological interventions to enhance stress resilience in order to prevent the development of addiction and other stress-related disorders.

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INTRODUCTION

The noun resilience derives from the Latin *resiliens*, the present participle of resilire (re- "back" + salire "to jump"), and was first used by Cicero and Francis Bacon (among others) as a synonym of rebound[1]. From the nineteen century on, material science has also used the word resilience to indicate the flexibility of a material or its ability to resist stress (force being applied) without permanent deformation. In the context of psychology, resilience can be defined as "the process of adapting well in the face of adversity, trauma, or other significant sources of stress"[2,3]. Besides the rebound of the equilibrium, resilience often implies an increase in mental resistance.

Although resilience is sometimes considered an extraordinary capacity of some individuals, research indicates that it is a common trait. The majority of individuals exposed to trauma or stressful events adapt to and overcome stress and maintain normal psychological and physical functioning without developing stress-related disorders[4]. Approximately 50% of people experience trauma in their life, but the prevalence of post-traumatic stress disorder (PTSD) is about 8%[5]. Resilience is an innate capacity, although it is not a stable trait, it is a dynamic process[6,7] that changes through a life span and can be enhanced by different factors.

RESILIENCE TO STRESS AND DRUG ADDICTION

Most research on resilience has focused on the biological and behavioral profile of individuals who are resilient to developing psychiatric illnesses such as depression and PTSD after exposure to stress. However, studies on resilience to the effects of stress on the initiation, maintenance and relapse to addictive disorders are very limited. In fact, almost all research regarding substance use disorders (SUD) has focused on risk; *i.e.*, the factors that predispose an individual to develop an addictive disorder. Vulnerability to the effects of drugs of abuse depends on multiple factors, including biologic factors such as genetic load, which are modified by life experiences and the environment in which the individual lives. Stressful experiences have a profound impact on the brain[8], for this reason, stress can increase vulnerability to addiction. Exposure to stress, especially in early life and adolescence, induces long-term modifications in the physiological response to stress, emotional reactivity, the brain reward system and cognitive processing, all of which contribute to the increased vulnerability to develop a SUD[9]. However, as commented on before, most people are resilient to stress. Consequently, only a small percentage of individuals that experience a traumatic event or are exposed to chronic stress develop an addictive disorder.

In recent years there has been an important impulse in the study of resilience to develop a SUD or an addictive behavior. In fact, until 2010, literature related to resilience and addiction was scarce, while in the last ten years the number of works on the subject has increased exponentially (Figure 1). Epidemiologic studies indicate a clear association between low resilience (often during adolescence) and the increment of addictive behaviors[10,11]. Resilience is a factor moderating the relationship between stress and alcohol use disorders (AUD)[12] and is strongly associated with a

Calpe-López C *et al.* Resilience to drug addiction

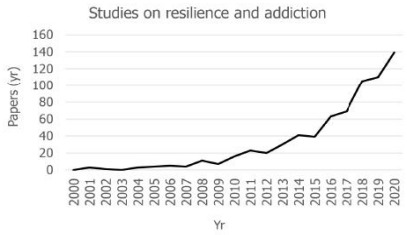

Figure 1 Results from the search "resilience and addiction" in the PubMed database (https://pubmed.ncbi.nlm.nih.gov, accessed on December 15, 2021). Number of papers published each year from 2000 until 2020.

reduction in the risk of AUD, though there is not a direct causal relationship but rather an overlapping of genetic and environmental influences[13]. Animal models used to study the impact of stress on drug addiction[14,15] are also being incorporated into research to identify the behavioral and physiological traits that characterize animals' resilience to the effects of stress on the rewarding properties of drugs of abuse, as well as the neurobiological substrates of the resilience process.

Addressing the perspective of resilience in the study of addictive behaviors is promising as a way of enhancing knowledge regarding the neuroscience of addiction. However, as Rudzinski *et al*[16] noted, there are difficulties in the use of the concept of resilience in the field of drug addiction, especially regarding its definition and operationalization as a trait, as a process or, as is more common, as an outcome (for example, the absence of SUD). In addition, it is important to distinguish between resilience (a concept with multiple meanings) and resiliency, which is a personality trait that has been linked to alcohol/drug problems and is defined as "the ability to flexibly adapt impulse control relative to contextual demand"[17]. Some studies define resilience as the capacity to maintain abstinence and not relapse to drug use during a recovery period[18,19]. In this sense, neuroimaging studies have shown that conserved prefrontal cortex (PFC) morphology and heightened neural PFC engagement are linked to abstinence and resilience against relapse in alcohol-dependent patients[19]. Other studies consider drug use as a stressor or risk factor for resilience (for example [20]), while some studies do not evaluate resilience to stress. In the last case, the concept of resilience is used to design a reduced response to the drugs of abuse in rodents that have been exposed to a genetic or pharmacological manipulation[21,22].

In the present review we mainly focus on research that has studied resilience to the effects of stressful experiences on subsequent drug use/abuse in animal models. First, we succinctly comment on the protective factors associated with resilience in studies with humans and explain the relationship between the physiological response to stress and resilience, since most human studies have focused on the neuroendocrine changes that are predictive of resilience. We then discuss the main animal models used to study resilience to social stress and review advances concerning the neurobiological substrates of resilience in said studies. Finally, we discuss research that specifically addresses resilience to the effects of repeated social defeat (SD) on the rewarding properties of drugs of abuse and lay out future research directions and conclusions.

BEHAVIORAL TRAITS AND PROTECTIVE FACTORS ASSOCIATED WITH RESILIENCE

Different protective factors associated with resilience can be identified on biological, psychological, and social levels. Among the internal factors are stable predispositions (such as genotype or personality traits) and the influence of skills or capacities acquired through interaction with stressors (emotion-regulation abilities, appraisal styles, *etc.*). Resilient people are more prone to experience positive emotions, realistic optimism, cognitive reappraisal (ability to replace negative thoughts with more positive ones), secure attachment, an active coping with stress, high coping self-efficacy, self-esteem, empathy, prosocial behavior and altruism, a healthy lifestyle (for example, physical exercise) and a sense of coherence (moral compass that gives

meaning of life)[4,16,23,24].

There are also external factors related with resilience at three levels[16]: Family (parental supervision, setting boundaries, bonding, support, *etc.*), school (positive environment, good relationships with teachers and peers, school engagement and extra-curricular activities involvement, *etc.*) and community (positive relationships with friends or neighbors, participation in religious practices, community engagement, community support, *etc.*). All these internal strengths and external resources help to prevent maladaptive responses to adversity[9]. Longitudinal studies have indicated several key factors related with resilience and a successful transition from childhood and adolescence to adulthood, such as social support (family, peer relationships, romantic partners, *etc.*), self-discipline, and good cognitive and executive functioning (planification, cognitive flexibility, *etc.*)[25]. Children exposed to war show increased risk of PTSD in adulthood, but some protective factors against the deleterious impact of war have been identified, including a loving and supportive environment (family, peers, teachers, *etc.*), a shared sense of values and religious beliefs, positive thinking and generosity[26]. Similarly, in patients with psychiatric disorders (depression and/or anxiety), factors predictive of low resilience include lack of purpose in life, less frequent physical exercise and low spirituality[27]. A study with fire-workers indicated that the trait of mindfulness (concentration on and moment-to-moment awareness of bodily activities and feelings) contributed to resilience, thus reducing avoidant coping in response to stress. Fire-workers with this trait reported less alcohol problems and reduced physical, depressive and PTSD symptoms[28].

As mentioned before, resilience is a dynamic process that raises individuals up from life's adversities and allows them to successfully overcome stressful events. The phenomenon known as "stress inoculation" occurs when a person exposed to mild or moderate stressors develops an adaptive stress response and shows a higher resilience to the negative effects of a variety of subsequent stressors[5,29]. As demonstrated by the group of McEwen, the behavioral effects of stress follow an inverted U–shape curve; low and high stress levels induced impairing effects, but intermediate levels promote better coping responses[30]. In the same way as a vaccine induces immunity against disease[2], stress inoculation is a form of immunity or protection against later stress that may be a result of neuroplasticity in the PFC[29]. The "Systematic Self-reflection model" proposes that engaging with moderate stressors can have positive consequences on mental health if scaffolded in self-reflection, a meta-cognitive skill (consisting of an honest reflection on the individual's coping and emotion regulatory practices) that leads to a cognitive maturity and on-going adaptation of the capacity of resilience[31]. On the other hand, substance use and other adjustment problems (depression, anxiety, rule-breaking, *etc.*) have been observed in adolescents from affluent families that have not been exposed to identified stressful experiences. These individuals are now considered as a group at risk that needs to build resilience through positive changes in parenting, construction and maintenance of supportive social networks, promotion of coping self-efficacy and self-esteem, *etc*[32].

PHYSIOLOGICAL RESPONSE TO STRESS AND RESILIENCE

Exposure to a physically or psychologically stressful stimulus immediately activates a physiological response characterized by a cascade of hormones in the hypothalamus-pituitary-adrenal (HPA) axis that prepare the body for fight or flight. The paraventricular nucleus of the hypothalamus releases corticotropin-releasing factor (CRF), which leads to the release of adrenocorticotropic hormone (ACTH) by the adenohypophysis, which in turn stimulates the release of glucocorticoids (cortisol in humans and corticosterone in rodents) by the cortex of adrenal glands. There are negative feedback mechanisms in the HPA axis; for example, glucocorticoids suppress CRF and ACTH production. In addition, stress activates the sympathetic nervous system (SNS), which induces the adrenomedullary release of noradrenaline (NA). Stress also stimulates the brain's noradrenergic system, resulting in the release of NA from the locus coeruleus (LC) to the amygdala, hippocampus, hypothalamus and PFC [33,34]. Dopamine (DA) release is also altered by stress, with an increase in the PFC and a reduction in the nucleus accumbens (NAcc)[35], and acute stress increases serotonin turnover in the amygdala, hippocampus, PFC and NAcc[36-38], although other studies have shown a lack of an effect of acute stress on serotonin turnover in the amygdala, NAcc, striatum[39] and hypothalamic paraventricular nucleus[38].

Glucocorticoid elevation may cause damage and atrophy of neurons in different brain areas involved in memory and emotional behavior, such as the hippocampus

and amygdala, inducing physical and psychological problems. Moreover, chronic stress interferes with the activity of neurotrophic factors that are responsible for the formation and strengthening of new neurons and synaptic connections, especially in the hippocampus, such as brain-derived neural factor (BDNF). The volume of this structure and the levels of BDNF are reduced in subjects exposed to prolonged stress, which could be a risk factor for the development of PTSD[40-42]. Resilience can avoid these negative effects of stress, for example, through the release of substances that block the physiological stress response. Neuropeptide Y (NPY) and dehydroepiandrosterone (DHEA) counteract CRF and cortisol, respectively[43,44]. Higher levels of NPY in response to acute stress predict less psychological distress and fewer symptoms of dissociation[45]. Furthermore, the brain of resilient people produces more BDNF, which also decreases levels of glucocorticoids in the hippocampus, and BDNF-mediated plasticity increases attention and memory and accelerates recovery from adversity. Resilient people have been shown to exhibit an adaptive stress response, rapid stress recovery (levels of cortisol decreasing fast after adversity) and lower susceptibility to stress-related physical and mental pathology[4].

There is an interface between the endocrine stress response and the immune system. Communication between neural, hormonal and immune systems is mediated by cytokines and chemokines, small molecules that mediate inflammatory processes, corticosteroids, pituitary hormones, catecholamines and neuropeptides[46,47]. Feedback between the peripheral immune system and the brain contributes to individual differences in the behavioral response to stress[48,49]. Resilient subjects display reduced neuroinflammation, which facilitates habituation to and recovery from stressful events and explains the lower incidence of medical and psychiatric diseases amongst these individuals[49,50,51]. Resilient people have lower systemic inflammation, and the psychosocial factors associated with resilience mitigate the impact of stress on systemic inflammation[51]. These bidirectional relationships between resilience and immunity are modulated by the gut microbiota[52]. There is an interaction between the gut and the brain that involves neural, endocrine, and immune pathways. It seems that the stress-induced activation of the HPA axis stimulates the immune system and causes changes in microbial diversity[53]. The gut microbiota has been associated with a wide range of physiological processes, including the response to stress[54]. Oral intake of Bifidobacterium was shown to significantly increase the number of mice that were resilient after repeated SD stress with respect to control animals not receiving treatment[55]. Moreover, administration of Lactobacillus was found to decrease anxiety-like behavior induced by repeated SD stress and to improve the immune response[56].

ANIMAL MODELS AND BEHAVIORAL PARADIGMS TO STUDY RESILIENCE TO SOCIAL STRESS

Animal models are necessary to understand the different aspects of human resilience, such as physiological or behavioral changes. As mentioned before, after exposure to stress, some humans develop a psychopathological disorder, such as depression or anxiety, while others are resilient to such effects. These disorders are complex and multifactorial and affect many aspects of human life; thus, no animal model can mimic the complexity of human disorder. However, animal models are useful for simulating some of the psychiatric symptoms[57] or behavioral dimensions that characterize a disorder[58]. After exposure to chronic stress, some animals develop depression– and anxiety–like symptoms and other behavioral alterations (susceptible or vulnerable animals), while others exhibit clear resistance to at least some of the maladaptive sequelae of stress (resilient animals). In addition, animal models also contribute to our understanding of the mechanisms underlying the development of resilience, such as the therapeutic effects of the inoculation of stress[59].

In this section, we first describe the animal models and behavioral tests used to study resilience to the symptoms of stress-related disorders, such as anxiety, depression, cognitive impairment or drug addiction and then the models used to induce stress in experimental animals. This is not an exhaustive review of these models, but only a brief description of the main paradigms used in preclinical studies of resilience. We focus on the model of SD stress in rodents, and on the behavioral paradigms that have been used to evaluate its short- and long-term consequences.

ANIMAL MODELS OF STRESS EXPOSURE

There are multiple techniques to induce stress in experimental rodents. Some of them use pharmacological stressors, such as daily administration of corticosterone[60], or physical stressors, such as restraint or immobilization[61]. Another model is based on a combination of physical and psychosocial stressors (chronic unpredictable stress (UCS) or chronic "mild" stress (CMS) paradigm)[62]. In the CMS, most animals (about 70%) show anhedonia–like symptoms (less sucrose consumption), reduction of hippocampal volume and alterations in glutamate metabolism, although there is a subset of resilient animals that do not exhibit these changes[62]. Resilience to stress has also been studied with the model known as "predator odor", in which the stress response is induced by exposing animals to the odor of a predator[63]. Usually, rats are classified into 3 groups according to the number and type of behavioral deficits observed as extremely, partially, or minimally disrupted. Anxiety–like symptoms, increased acoustic startle responses and reductions in NPY are observed in animals that are extremely disrupted, while partially and minimally disrupted animals exhibit mixed deficits within these domains[63].

The paradigm of learned helplessness is an animal model of depression that is also employed to induce stress and study resilience by exposing animals to the stress induced by an inescapable, unpredictable and uncontrollable foot shock[60,64,65]. After such exposure to stress, a subset of susceptible animals develops learned helplessness (coping deficits to deal with the inescapable shocks), while another subset of resilient animals displays escape responses with latencies similar to those of non-stressed animals[64]. Results are in function of the severity, duration and control over cessation of the footshock, the last variable of which promotes resilience[65].

As commented on before, in the present work we focus on the model of chronic SD stress because it is the most used animal model to study resilience to the effects of stress and has more ethological and ecological validity. In fact, the most frequent type of stress faced by humans is the chronic social stress derived from problems with social interaction (family or friend relationships, work-place stress, bullying, *etc.*). In the chronic SD model, brief episodes of aggression from a more aggressive conspecific in the resident-intruder paradigm result in the defeat of the experimental animal (intruder), which usually shows anxiety- and depression-like symptoms[15,66-69]. In the most widely employed SD model, rats or mice are exposed to SD for 10 days. Each day, the experimental animal undergoes 10 min of physical attack by the aggressive opponent, followed by 24 h of sensory contact. The consequences of this kind of stress are also a function of the severity and duration of the defeat episodes but chronic SD exposure induced an escalation of cocaine and alcohol consumption. To study resilience, genetically inbred C57BL6/J male mice are usually employed. Following chronic SD stress, all mice exhibit heightened reactivity of the HPA axis, deficits in exploration (interpreted as increased anxiety) and polydipsia[70]. However, there are differences between susceptible and resilient mice regarding other consequences of chronic SD. Resilient mice do not exhibit social avoidance, hyperthermia elicited by social interactions, anhedonia–like symptoms, or metabolic syndrome, characterized by over–eating, obesity, and leptin resistance[70,71]. Approximately 35% of C57BL6/J mice are resilient, although the relative distribution of resilience differs across strains [72]. Similarly, wild-type Groningen rats have better coping strategies and are more resilient to SD stress than Wistar rats[73].

A variation of the classical 10-day SD paradigm consists of exposing animals to intermittent repeated SD (IRSD); usually, four episodes of defeat separated by intervals of 72 h. The IRSD model is frequently employed in studies on the influence of social stress on vulnerability to developing drug addiction. Exposure to IRSD has also been shown to increase the rewarding effects of drugs of abuse[14,74,75,76]. In our laboratory, mice exposed to IRSD during adolescence or adulthood exhibit a long-term enhanced sensitivity to the rewarding effects of drug of abuse such as cocaine and MDMA[77,78,79].

To study the phenomenon of "stress inoculation" several types of moderate stressors have been used, including exposure to intermittent foot shocks[80] and brief intermittent maternal separations during early periods of life[81] or a combination of maternal separation and UCS[81]. Infant rats exposed to intermittent foot shocks subsequently respond more effectively than non-stressed control rats when confronted with novel situations[80]. The combination of maternal deprivation during early life with UCS during adolescence promotes greater resilience in adulthood than maternal deprivation alone or when combined with UCS[81].

During chronic exposure to stress, behavioral strategies that limit the experience of stress may promote resilience[5]. During chronic SD, animals that engage in less submissive postures when threatened and attacked by the opponent show less social avoidance, suggesting that this behavioral coping strategy reduces the effects of the stress[82]. Behavioral manipulations have also been used to reduce the effects of stress and increase resilience; for example, exposure to physical exercise[83,84] or environmental enrichment[85].

BEHAVIORAL PARADIGMS TO STUDY STRESS-RELATED PSYCHIATRIC DISORDERS

Behavioral tests of anxiety- and depression-like symptoms

The forced swim test (FST) is a classic behavioral test of depression-like symptomatology in which animals are placed into a cylinder filled with water and forced to swim during a period lasting a few minutes. Initially animals attempt to escape and swim, but afterwards they stop fighting and become passive. Immobility (passive floating with minor movements necessary to keep the head above water) is interpreted as a failure to persist in escape-directed behavior, hopelessness, negative mood and depressive-like behavior. The FST is frequently used to evaluate resilience since SD increases immobility in this test in susceptible but not in resilient animals[60,86,87,88]. Similar to the FST, the tail suspension test (TST) measures immobility, which is considered to represent despair and depressive-like behavior[89]. Rodents are hung in an uncontrollable fashion by their tail for a few minutes[90] and, after initial escape-oriented movements, develop an immobile posture. The effects of SD exposure in the TST are unclear, and it has been suggested that this paradigm models the stress-coping strategy from which depressive-like behavior is inferred[91]. An increase in immobility is observed in animals reared in a limited bedding and nesting environment, which induces erratic maternal care and social stress[92]. Similarly, exposure to chronic mild stress (CMS) has been shown to increase immobility in anhedonia-susceptible animals[87]. However, our group has recently observed a reduction of immobility after IRSD exposure, which could be interpreted as an enhanced reactivity of defeated mice to the situation of moderate inescapable stress that the TST represents [93].

Anhedonia– or depressive-like symptoms are also frequently evaluated by measuring sucrose consumption. During training, after some hours of food and water deprivation, a bottle containing a sucrose solution is made available in the home cage. Sucrose intake is measured at different intervals during stress exposure and is reduced in vulnerable but not resilient stressed animals[70,71,88,94,95]. The splash test consists of spraying a 10% sucrose solution on the dorsal coat of a rodent to stimulate grooming behavior. An increase in the latency of grooming and a decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior[96]. This test has also been used to evaluate resilience to the consequences of SD stress[60,93].

In the social interaction test, animals are placed within an open field in two trials (2.5-10 min), in the absence (no target) and presence (target) of a conspecific animal contained in a perforated Plexiglas cage, in order to allow for social interaction while preventing confrontation. Social avoidance is considered to take place when the experimental animal spends less time in the area immediately surrounding the enclosure containing the opponent (interaction zone) and more time in the corners of the open field. Social avoidance is associated with depressive-like behaviors and is frequently observed after SD exposure in susceptible but not in resilient animals[70,71,93,97,98].

The novelty suppressed feeding test is based on the innate fear of rodents of novelty and the inhibition of feeding behavior when exposed to a novel environment. Animals' access to food is restricted for 12-24 h. Animals are placed in a corner of a box containing a pellet of food and the latency to begin eating is recorded. Immediately after this, animals are placed in its home cage and the amount of food consumed in 5 min is measured. This test detects behaviors related to depression and anxiety, because a conflict appears between the anxiogenic environment and hunger-induced behavior[60,99].

The elevated plus maze (EPM) is one of the most used paradigms to measure anxiety in rodents. This test is based on the natural aversion of rodents to open elevated areas and the exploratory behavior that they exhibit in novel environments. The apparatus, elevated about 50 cm above floor level, consists of two open arms and two enclosed arms, and the junction of the four arms forms a central platform. Subjects

are placed on the central platform and allowed to explore the maze for 5 min. The total time spent in and the number of entries into the open (and closed) arms, and the percentage of time and entries into the open arms are measured. Anxiety levels are considered to be lower when the measurements in the open arms are higher and those in the closed arms are lower, and vice versa[100,101]. Mice exposed to chronic SD exhibit higher anxiety levels in this paradigm[93,97,102]. The EPM is also frequently used in studies of resilience to the effects of social stress on anxiety[60,92,102]. In a recent study in our laboratory, we observed that mice that were resilient to the effects of stress on cocaine reward spent less time in the open arms[93].

In the open field/exploration test, the animal is placed into an open-field arena for several min and its locomotor activity is evaluated by measuring distance travelled and velocity. A reduction of these measures is indicative of anxiety[103]. Sometimes the open field is divided into a center and a surrounding area, with thigmotaxis being indicative of anxiety. Maternal separation decreases the time that mice remain in the center of the open field[104]. SD induces deficits in exploration that are not observed in resilient animals[60,70,71,102].

The hole-board test is used to evaluate anxiety-related and novelty-seeking behavior of rodents. This test is carried out in a square box with equidistant holes in the floor. The animal is placed in a corner of the box and is allowed to freely explore the apparatus for a few minutes. Head-dipping represents exploratory tendencies that are distinct from general locomotor activity; thus, the latency to perform the first head-dip and the frequency of dips is recorded. Stress exposure elevates anxiety-related behavior in the hole-board test in rats and mice[105,106]. In our laboratory, we have observed that mice with low novelty-seeking are resilient to the effects of SD on cocaine reward[93].

Behavioral tests to evaluate cognitive impairment

The novel object recognition test evaluates episodic memory in rodents[107] and has been used to measure cognitive dysfunction according to deficits in object-context identification[108]. The task is performed in an open field box and consists of three phases: habituation (free exploration of the empty box), training (exploration of the box, which contains two small river stones) and test (one of the stones is replaced with a small plastic toy). In the training and test sessions, separated for a memory retention interval (1 min), the exploration of the objects is measured for 3 min. It is assumed that if the animal recognizes the stone, it has spent more time exploring the new object. Exposure to different paradigms of stress induces cognitive deficits in recognition memory[94,104,105,109]. Acute[110] and chronic[97] SD impairs performance of the object recognition task. This task has also been used to study resilience to the impairing effects of social stress on cognition[88,94,97,111,112].

The Morris water maze task measures spatial memory that is dependent on the hippocampus[113]. The apparatus consists of a circular swimming pool, divided into 4 equal quadrants (NW, NE, SE and SW), with an escape platform placed 1 cm below the water surface. Several visual cues surrounding the maze are placed on the walls. During the training phase the animal is placed in the water inside one of the quadrants and allowed to swim freely until it locates and climbs onto the platform. If the animal fails to locate the platform, it is guided to the platform by the experimenter and allowed to stand on it for several seconds. The training is performed over 4-5 consecutive days (3 trials per day), measuring the escape latency (the time taken to locate the platform in each trial). The test is performed 24 h after the last training session (the platform is removed and the time spent in each quadrant is measured). If the animal recalls the placement of the hidden platform it will spend more time in that quadrant. Unpredicted CMS impairs performance of the water maze[114], but chronic SD stress does not affect this task[102]. The water maze has also been used to study resilience to the effects of stress on cognitive processes[114,115]. An interesting study [115] showed that rats that emitted ultrasonic vocalizations during intermittent swim stress later showed resilience in the Morris water maze and an instrumental swim escape test.

The Y-maze is a spatial task that requires intact hippocampal function[116]. The Y-maze apparatus has three identical and symmetrical arms that radiate out from the center. Explicit cues are presented outside the maze (located on the walls around the room). In the first trial, the animal is placed in one arm, designated as the "start" arm, while another arm is blocked so that the animal can only explore the start and the other arm. After 4 h, in the second trial, animals are placed in the start arm and can freely explore all three arms. The number of entries and the time spent in each arm is measured. If the animal recalls the arms previously explored in trial 1, it will spend more time in the "novel" arm in trial 2 (discrimination performance). CMS induces

deficits in the performance of the Y-maze among vulnerable anhedonic-rats[88]. Acute [110] but not chronic[117] SD stress also impairs performance in the Y-maze. However, the combination of chronic SD with a slight peripheral infection (produced by injection of a sub-threshold of LPS) impairs the performance of susceptible mice in the Y-maze [111].

The radial arm maze is a model of hippocampus-dependent memory. Animals are food-restricted (approximately 85.0% of their previous body weight) and pre-trained to associate the maze with a food reward placed at the end of all 8 arms. Subsequently, the animals are trained for several consecutive days. In each trial the animal is placed in the central chamber of the maze for habituation and can then freely explore the arms until it consumes all food reward or until a maximum time. The measurement of memory is the number of errors committed, defined as entries in a previously visited arm[118,119]. Chronic stress induced by visual and olfactory exposure to a predator (Long Evan rat) without direct physical contact impairs performance in the radial maze[118]. Similarly, maternal separation induces an overall impairment in the performance of the radial maze in adulthood; however, this impairment is observed in susceptible, but not in resilient mice[119]. On the other hand, adult rats exposed to maternal deprivation perform better in the radial maze, an effect probably related with the phenomenon of inoculation of stress[120].

The radial arm water maze also evaluates spatial ability in rodents[121,122,123]. In this case, the radial arm maze is filled with cloudy water to conceal a platform placed in one of the eight arms, and there are prominent extra-maze cues on the walls of the room. The animals perform several trials in three days, which consists of placing the animal into an arm (the start arm, which does not contain the platform). When the animal reaches the hidden platform it remains on it for several seconds to visualize the room spatially. If the animal fails to find the hidden platform, it is guided there by the experimenter. The number of entrances is measured in each trial. Two types of errors are considered in each trial; reference memory errors (number of first-time entries into arms that did not contain the platform) and working memory errors (number of repeat entries into an arm that did not contain the platform). Chronic restraint stress impairs radial arm water maze performance[122,123], but this effect recedes with time[124] and is prevented by environmental enrichment[85].

It is important to note that chronic stressors do not affect the performance of females in most of these tests (spatial object recognition, radial arm maze, Morris water maze, Y-maze), while males show stress-induced impairments in all of them[125]. These sex-dependent differences include the use of different strategies by the sexes to solve cognitive tasks and may be related to estradiol levels[87].

Animal models of addiction-like symptoms

The animal models of drug reward and addiction-like symptoms are essential to progress in understanding the biological basis of SUD and for the identification of new therapeutic targets. Drug addiction is a neuropsychiatric disorder characterized by loss of control over drug-seeking and drug-taking, the presence of a negative emotional state and an intense craving for the drug when it is not available, and a high propensity to relapse even after long-term periods of abstinence[126]. Drug addiction represents a profound disruption of different neural circuits, including a deficit of the brain reward system, an over-activation of the stress systems, aberrant associative learning (which confers exaggerated incentive salience to stimuli or contexts associated with the drug), and a dysfunction of the PFC, resulting in the inability to inhibit drug-taking behavior. The transition from an initial recreational and controlled drug use to compulsive consumption is also related with a change from the ventral to the dorsal striatum in the control of drug use behavior, with the consequent development of rigid stimulus-response habits[127,128].

Drug addiction has a multifactorial nature, since environmental and biological factors interact to confer vulnerability or resilience to the development of this disorder. The complexity of addictive behavior cannot be captured by an animal model, but they are useful in modelling some specific aspects of drug addiction. The two main models to study vulnerability or resilience to drug addiction are the self-administration (SA) paradigm, which is based on the primary hedonic effects produced by the consumption of a drug of abuse, and the conditioned place preference (CPP) paradigm, which focuses on the component of reward related to associative or incentive learning.

The intravenous SA paradigm is the most important procedure for assessing the primary intrinsic reinforcing effect of drugs, and is the most commonly used in rodents[129,130]. In this paradigm animals are trained in daily sessions to obtain the drug by performing an operant response; for example, by pressing a lever or performing a nose-poke. This response is reinforced by injection of the drug, usually

according to a fixed response (FR) program in which the animal must perform a fixed number of responses in order to obtain the dose of the drug. Variable or progressive response programs are also used to measure motivation of the animal for the drug. The oral SA paradigm, frequently used for alcohol, is similar regardless of the way in which the substance is ingested by the animal. Pharmacological and methodological factors may influence the results obtained with the SA paradigm, such as the drug, dose and rate of infusion, duration of the SA session, the requirements of response, the sex and age of the animal, *etc*.

The SA paradigm is also used to study extinction and reinstatement of drug-seeking behavior. During the extinction phase, the drug of abuse is not presented after responding, and as a consequence, a progressive decrease in the operant response takes place[131-133]. When extinction has been completed, reinstatement of the operant response by several stimuli is observed. Reinstatement of drug SA is a model of relapse to drug consumption after a period of abstinence. As in humans, administration of the drug of abuse (priming), re-exposure to drug-associated stimuli, or exposure to stress reinstates the initially learned operant response[134]. Indeed, some researchers have adapted the SA paradigm in order to model the main features of addiction in humans based on the DSM-5 criteria: loss of control or persistence in drug seeking (active responses during periods in which the reinforcer is not available), high motivation for the drug (using a progressive reinforcement schedule), and maintenance of consumption despite its negative outcomes (association between reinforcement and a foot shock)[135]. The SA model has excellent predictive and face validity; however, it also has some drawbacks related with the complexity of the technique (surgical implantation of an intravenous catheter or previous familiarization with the drug for intravenous or oral SA, respectively) and the training of the animals until they effectively acquire operant response.

Using the SA paradigm, it has been demonstrated that exposure to social stress increases the reinforcing effects of drugs of abuse[136-139]. Recently, resilience to these effects has also been studied using different types of social stress and drugs of abuse, such as cocaine[140-143], methamphetamine[144] and alcohol[145-147].

The CPP is a paradigm that evaluates the conditioned rewarding effects of a drug of abuse, since some contextual stimuli acquire appetitive properties when associated with the drug[148-151]. This paradigm is characterized by its methodological simplicity and is thus frequently used. Animals are conditioned in a box with two or three compartments that are clearly distinct in terms of the stimuli present in each compartment; for example, they have different colored walls and floor textures. Before conditioning, a pre-conditioning phase takes place to evaluate the time spent by the animal in each compartment without any treatment. During conditioning the animal receives the drug (usually 4 injections in 4 or 8 days) in a specific compartment (without access to the other compartment) and physiological saline in the opposite compartment. Later, in the post-conditioning phase (equal to pre-conditioning) it is evaluated whether the animal has learned to associate the rewarding effects of the drug with the environmental cues present in the drug-paired compartment. If the animal spends more time in this compartment (in comparison to the time spent in pre-conditioning or to the time spent in the saline-paired compartment), it is considered that the animal has acquired CPP. All drugs abused by humans induce CPP in rodents [150].

As described for the SA paradigm, the CPP procedure can also be used to evaluate other processes besides acquisition, such as extinction and reinstatement of motivated behavior[148]. To induce extinction, animals are placed in the CPP box and perform daily or weekly sessions similarly to pre- and post-conditioning (*i.e.*, they are exposed to the previously drug-paired compartment without administration of the drug). Progressively, the association between the reinforcing value and environmental cues weakens, and the CPP is finally extinguished. The period needed for extinction of CPP is influenced by different factors, including exposure to stressful events. For example, exposure to SD before each acquisition session[152], or 3-weeks before the initiation of the CPP procedure[78], slows the extinction of MDMA-induced CPP. After extinction, an injection of the drug of abuse (priming) or exposure to stress induces the reinstatement of CPP. In this paradigm, reinstatement refers to the recovery of the conditioned response and involves renewed memory of the association - learned during conditioning - between the reinforcing effect of the drug and the environmental cues associated with its pleasant effects. In our laboratory we have observed that SD exposure induces reinstatement of the CPP induced by cocaine[153,154].

The CPP has been widely used to evaluate the influence of social stress on the conditioned rewarding effects of different drugs of abuse, including alcohol, cocaine and MDMA[15]. In our laboratory, the animals are exposed to SD three weeks before

initiation of the CPP procedure. We have seen that exposure to SD induces a long-term increase in the rewarding effects of cocaine, since defeated mice acquire CPP with doses that are ineffective in inducing place conditioning in control mice[77]. Furthermore, we have observed how SD induces a long-term enhancement in the vulnerability of mice to priming-induced reinstatement of the CPP induced by cocaine [155] and MDMA[78]. In addition, the CPP model has been used to study resilience to the effects of social stress on the rewarding effects of methamphetamine[156], MDMA [157] and cocaine[93,143,158,159,160-163].

Finally, the effects of social stress on alcohol intake and resilience to these effects have been studied in the two-bottle choice test, a paradigm of voluntary consumption, in which animals can choose freely, during a limited time, to drink from one of the two bottles placed in the home cage: one containing water and the other containing alcohol [92,164].

ADVANCES CONCERNING THE NEUROBIOLOGICAL SUBSTRATES OF RESILIENCE

The study of the neurobiology of resilience is a relatively young area of scientific investigation[24,35]. Research carried out with animal models in the last decade has identified several behavioral, hormonal, neural and molecular mechanisms underlying the development and enhancement of resilience, mainly in relation with the reduced susceptibility to develop psychiatric disorders, such as depression or PTSD, after stress or trauma (Figure 2). As Russo *et al*[5] noted, resilience is mediated not only by the absence of neurobiological abnormalities that occur in susceptible animals after stress exposure (passive resilience), but also by the presence of neuroadaptations which occur in individuals that are resilient to stress, which help them to maintain normal functioning (active resilience). In this section we review the main results obtained in studies using electrophysiological, optogenetic, pharmacological, and molecular profiling techniques to unravel the neurobiological substrates of resilience to the negative consequences of chronic SD stress, mainly social avoidance and anhedonia. Advances in this field may guide ongoing research regarding the neurobiological substrates of resilience to the effects of SD on addiction disorders.

Glutamatergic system

The glutamatergic system seems to play an important role in resilience to stress[165]. Chronic stress reduces the dendritic spine density of glutamatergic neurons in the PFC and hippocampus, while it increases it in the amygdala and NAcc[166]. In the chronic predator and SD stress paradigms, resilient mice show greater expression of immediate early genes (c–Fos, FosB, or ΔFosB) in glutamatergic neurons of the medial PFC[106,167,168] and in medium spiny neurons (MSN) of the NAcc, inducing expression of the AMPA glutamate receptor subunit GluA2[169]. Optogenetic stimulation of either medial PFC or amygdala glutamate afferents to the NAcc induces resilience[168,170], while attenuation of glutamatergic transmission from the ventral hippocampus to the NAcc is pro-resilient, and reduced activity in the ventral hippocampus is observed in mice that are resilient to the effects of chronic SD[170]. Furthermore, several environmental manipulations that promote resilience to stress–induced depression– and anxiety–like behaviors, such as early intermittent maternal separation and environmental enrichment, increase the volume of ventro-medial PFC[171], the dendritic spine density of hippocampal and PFC neurons[172], and expression of FosB and ΔFosB in medial PFC[167]. All these results suggest that increased neuronal activation of mPFC represent pro–resilience adaptation[5].

NMDA receptors have been implicated in stress resilience[165]. Mice susceptible to chronic SD stress exhibit low activity of hippocampal extrasynaptic NMDA receptors, and enhancement in the function of these receptors prevents social avoidance behavior in defeated mice[173]. The NMDA antagonist ketamine protects against the long-term consequences of different types of stress in animal models[165,174]. For example, administration of ketamine protects mice against SD-induced depressive sympto-matology in the FST and against learned helplessness-induced coping deficits when dealing with inescapable shocks, although it did not protect against the anxiety-like phenotype in the EPM[60]. Reducing brain D-serine, an endogenous co-agonist at the glycine site of the NMDA receptors, may also improve stress resilience[175], and NMDA receptor blockade in the right medial PFC facilitates resilience to SDS-induced anxiety in mice[176]. Furthermore, we have observed that the NMDA antagonist memantine increases resilience to the effects of IRSD on the CPP induced by cocaine in

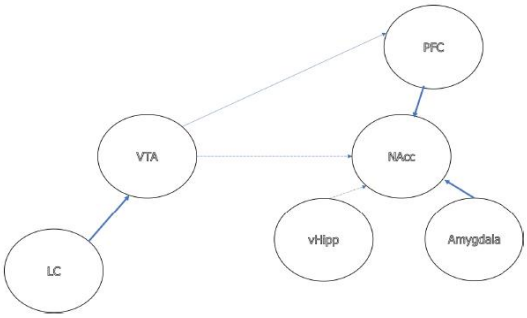

Figure 2 Simplified diagram of the neurobiological substrates of resilience to the effects of social defeat in rodents. Resilience is induced by activation of the pathways indicated with gross lines and by inhibition or normalization of the pathways represented with dashed lines. LC: Locus coeruleus; VTA: Ventral tegmental area; PFC: Prefrontal cortex; NAcc: Nucleus accumbens; vHipp: Ventral hippocampus; Amyg: Amygdala.

mice[77].

Some subunits of AMPA receptors might be involved in resilience. For instance, mice resilient to developing social avoidance after chronic SD show increased GluR2 mRNA expression compared to control mice, while susceptible mice display a decrease in GluR2 levels in the NAcc[169]. In addition, AMPA agonists prevent increases in corticosterone and latency to feed in the novelty-suppressed feeding induced by chronic stress[177].

The role of metabotropic glutamate receptors in stress resilience remains uncertain [165]. After 3 days exposure to learned helplessness or SD, mGluR5 KO mice exhibit enhanced susceptibility to stress-induced depression, social avoidance, and anhedonia. In addition, susceptible mice exhibit less mGluR5 in the NAcc than both resilient and control mice[178]. Finally, blockade of mGlu2/3 and deletion of mGlu2, but not mGlu3, promotes stress resilience, including protection against stress-induced depressive-like symptoms[179].

GABAergic system

There are a limited number of studies on the role of GABA in resilience to the effects of chronic SD, and the effects observed to date have been in the function of the brain area containing GABA neurons and the subtype of receptor studied.

Chronic SD defeat activates GABA neurons of the dorsal raphe nucleus (DRN) and strengthens inhibition of 5-HT neurons in susceptible mice, but this effect is not observed in resilient mice without a social interaction deficit; accordingly, optogenetic inhibition of DRN GABA neurons was shown to disinhibit 5-HT neurons and promote resilience[180]. Conversely, SD stress impairs the inhibitory tone in the NAcc. Stress-susceptible mice exhibit reduced levels of inhibitory synaptic markers and protein expression (vesicular GABA transporters (vGAT) and gephyrin) in the NAcc that are not observed in resilient mice[181]. GABA (B) receptors in the habenular nuclei are also down-regulated in susceptible mice, which display elevated c-Fos expression in this structure; furthermore, intra-habenular injection of baclofen and CGP36216 (GABA (B) agonist and antagonist, respectively) reverses social avoidance[182]. Studies with KO mice have also indicated the role of GABA in resilience to the effects of SD. GAT-1-deficient mice demonstrate an increase in resilience to the effects of acute stress on depressive- and anxiety-like symptoms[183,184]. Moreover, GABA(B1a) KO mice are more susceptible, whereas GABA(B1b) KO mice are more resilient to both stress-induced anhedonia and psychosocial stress-induced social avoidance[185].

Dopaminergic system

Adaptations within the brain reward system, and in particular in the mesolimbic DA circuit, are closely associated with resilience to the effects of chronic SD stress. The firing rate of ventral tegmental area (VTA) DA neurons has been shown to be increased in susceptible animals exposed to chronic SD; conversely, resilient mice show an increase in K + channels that normalizes hyperexcitability of VTA DA

neurons and prevents social avoidance and sucrose preference deficit[70,186,187]. A further increase in the hyperactivity of VTA DA neurons in susceptible mice produced by optogenetics or pharmacological treatments induces homeostatic plasticity and reverses depression-related behaviors[187,188]. Such studies bring to light the self-stabilizing capacity of midbrain DA neurons of the brain reward system[187]. A recent study has demonstrated that a baseline level of physical activity (voluntary wheel running), mediated by the tyrosine hydroxylase (TH) neurons in the VTA, affects susceptibility and resilience to chronic SD. Mice with low levels of physical activity showed lower TH expression in the VTA and were susceptible to SD, while mice with high levels of activity showed higher TH expression and were resilient to SD; activation of TH neurons in the VTA of mice with lower levels of activity increased resilience, while inhibition of these neurons increased susceptibility to SD[189].

Different MSN subtypes of the NAcc (D1-MSN and D2-MSN, with predominant expression of DA D1 and D2 receptors, respectively) are also involved in susceptibility and resilience to chronic SD stress. Susceptible mice that develop depression-like behaviors after SD showed decreased frequency of excitatory synaptic input in D1-MSN (but an increase in D2-MSN); in addition, enhancing the activity[190] or the spine density[191] of D1-MSN has been shown to induce resilience. Fosb-targeted histone methylation in D1-MSN or histone acetylation in D2-MSN promote a susceptible, depressive-like phenotype, while histone acetylation in D1-MSN or histone methylation in D2-MSN increase resilience[192]. Resilient animals also display an upregulation of synaptic strength at dendritic spines of D1-MSN and a concomitant downregulation in D2-MSN[193]. In addition, chronic SD selectively reduces NLGN-2, a neuronal postsynaptic cell adhesion protein, in DA D1-MSN of susceptible mice [181]. D1-MSN activity prior to stress is also a predictor of resilience, as mice that will later become resilient display increased baseline D1-MSN activity[194].

Single and repeated SD stress induces D1 receptor-mediated changes in medial PFC neurons. A single SD was shown to increase arborization and the spines of apical dendrites of pyramidal neurons in the medial PFC, whereas repeated SD reduced dendritic lengths of these neurons[195]. Optogenetic inhibition of the DA VTA neurons projecting to the medial PFC promotes susceptibility[188]. DA D1 receptors in medial PFC excitatory neurons plays a role in suppressing susceptibility to stress, since repeated SD reduces the expression of these receptors in susceptible mice, while its genetic deletion facilitates the induction of social avoidance[195].

DA transmission in other brain areas is also involved in susceptibility or resilience to stress, although results are contradictory. Vulnerable mice were reported to display increased expression of DA D2 receptors in the amygdala[102,196] and increased levels of DA in the hippocampus and PFC[197]. However, another study found that hippocampal dopaminergic activity was inversely correlated with the level of social avoidance induced by SD and chronic treatment with hop bitter acids enhanced stress resilience[198]. Similarly, treatment with caffeine (from 14 days before until the end of SD) reverses social avoidance and anhedonia, and this pro-resilience effect of caffeine is reversed by the antagonism of DA D1 (but not D2) receptors[199].

Noradrenergic system

Noradrenergic (NA) neurons in the LC have direct connections within the VTA and regulate vulnerability to SD through inhibitory control of VTA DA neurons[200]. NA LC neurons projecting to the VTA exhibit enhanced firing activity in resilient, but not susceptible, mice, and optogenetic activation of LC neurons in susceptible mice reverses depression-related behaviors[201]. α1- and β3-adrenergic receptors are highly expressed in VTA neurons projecting to the NAcc, and the antagonism of these receptors blocks the effects of the optogenetic and pharmacologic activation of LC neurons; *i.e.*, it reverses hyperactivity and homeostatic plasticity in the VTA-NAcc pathway in susceptible mice[201].

Serotonergic system

Plasticity of the serotonergic system also contributes to susceptibility or resilience to the effects of SD stress, although the role of serotonin depends on the brain area under consideration. As commented on before, inhibition of GABA neurons of DRN disinhibits 5-HT neurons and promotes resilience to social avoidance induced by SD in mice[180]. In fact, the mechanism underlying SD-induced social avoidance is a hyposerotonergic state in the DRN, which results from the activation of p38α mitogen-activated protein kinase (MAPK), the consequent translocation of the SERT to the membrane, and the increase in the rate of serotonin uptake[202]. Down-regulation of the 5-HT1A auto-receptors in 5-HT neurons of DRN (which can result in increased 5HT release), improves behavioral resilience to SD[203]. On the other hand, rats

susceptible to stress-induced anhedonia, but not resilient rats, display an increased number of neurons expressing tryptophan-hydroxylase-2 (TPH2, the enzyme for serotonin synthesis) in the ventral subnucleus of the DRN (DRNv), while activation of the CRF containing neurons of the amygdala induce resilience, suppressing the increase of TPH2 positive neurons in the DRNv and ameliorating anhedonia in susceptible rats[204]. Mice resilient to the effects of chronic SD also display a reduction of serotonin in the hippocampus[197].

Cholinergic system

ACh signaling in the hippocampus may be related with differential responses to SD stress. Interference with hippocampal AChE activity increases anxiety- and depression-like behaviors and decreases resilience to repeated SD stress[205]. In addition, nicotinic cholinergic (nACh) signaling in the basolateral amygdala seems to play a role in the effects of SD, since β2 nAChR subunit knockdown undermines resilience to SD stress and c-fos immunoreactivity in this structure[206].

Endogenous opioids

Chronic SD stress increases μ and κ opioid receptors and reduces δ opioid receptors in the PFC of susceptible mice (with social avoidance), while resilient mice show no alteration in the levels of opioid receptors and increased p38 MAPK phosphorylation [207]. Besides the increased mRNA expression of the opioid μ and κ receptors in the frontal cortex, susceptible mice also show a reduction in the expression of μ receptors in the hippocampus and a reduction of κ receptors in the basolateral amygdala[208, 209]. Conversely, mRNA of dynorphin is increased in the shell of NAcc in susceptible rats and in the striatum of resilient animals[208].

Chronic SD also decreases mRNA levels of δ opioid receptors and enkephalins in the basolateral amygdala and in the ventral hippocampus (CA1) of vulnerable mice [209]. Administration of an agonist of δ receptors increases resilience and reduces oxidative stress markers in CA1 neurons, a mechanism that may be involved in the pro-resilient effect of enkephalin signaling[210]. Similarly, susceptible animals display reduced enkephalin levels in the NAcc and enkephalinase inhibitors, while intra-NAcc infusion of a δ receptor agonist induces resilience and increases phosphorylation of extracellular signal-regulated kinase (ERK), which is downregulated by SD stress[211].

μ-opioid receptor G-allele carriers express less submissive behavior and exhibit resilience to SD, demonstrated by a lack of subsequent social avoidance and reductions in anhedonia; moreover, the resilience in question was associated with a greater induction of c-fos in the NAcc and periaqueductal gray[212].

Neuropeptide Y

Neuropeptide Y (NPY) is a neuropeptide that is widely distributed in the brain and promotes protective responses in the face of stress[213,214] by inducing anxiolytic effects and counteracting the anxiogenic effects of CRF. Multiple studies indicate a positive correlation between NPY levels and resilience to the deleterious effects of stress in humans and animal models. A significant down-regulation of NPY in the amygdala and hippocampus has been observed in animals with PTSD-like symptoms, and administration of NPY reversed the negative behavioral effects of predator-scent stress[63]. Mice susceptible to the effects of chronic SD also show a down-regulation of NPY and NPY2R in the hippocampus[215]. Administration of NPY significantly reduces submissive/defensive behaviors in socially defeated hamsters, although this effect is not mediated by the Y1 receptor[216]. Such results demonstrate that NPY may function as an important factor in resilience against the impairing effects of SD, and a recent study has suggested that deficiency of NPY plays a role in the impairing effects of stress on hippocampal function and the processes mediated by this structure[217].

Orexins

Orexins (OX) produced in the lateral hypothalamus also play an important role in the response to stress[218,219]. Chronic SD stress-susceptible and -resilient mice (with or without deficits in social interaction) display different levels of prepro-OX in the hypothalamus[220] and basolateral amygdala, with increased OX1 and decreased OX2 observed in susceptible mice[221]. Brain infusion of OX A was found to induce an antidepressant-like effect only in susceptible mice, while co-infusion of OX A and B induced an anxiogenic effect only in resilient mice[220]. In addition, knocking down the OX2 receptors in the basolateral amygdala increases social avoidance and reduces the time spent in the center of an open field[221]. Similarly, after SD stress, resilient (actively coping) rats express lower prepro-OX mRNA levels than passively coping

rats, while inhibition of OX before each SD episode increases social interaction and decreases depressive-like behavior in vulnerable rats[222]. These results suggest that lower levels of OX contribute to resilience to repeated SD, although in this context it is important to consider the different types of OX receptors. A recent study indicated that OX1 and OX2 receptors exert opposite functions and that the agonism of OX2 receptors promotes resilience to the anxiety and depression induced by exposure to SD stress in mice[223,224].

Neurotrophic factors

Neurotrophic factors and their signaling pathways, such as BDNF or ERK1/2, have been implicated in the neuroadaptations that take place in response to stress.

ERK is reduced after SD stress in both susceptible and resilient mice[207]. SD also decreases phosphorylation of ERK[211] and the pERK/ERK ratio[225]. Overexpression of ERK2 in the VTA increases susceptibility to SD stress in mice, while blockade of VTA ERK2 activity promotes behavioral resilience and decreases the frequency of firing of the VTA DA neurons, an important electrophysiological hallmark of resilience [226]. Phosphorylation of ERK is enhanced by treatments that induce resilience, such as the intra-NAcc infusion of a delta opioid receptor agonist or enkephalinase inhibitors[211].

BDNF is expressed in the amygdala, hippocampus, PFC and basal forebrain, and acts through its two main receptors, TrkB and p75[227]. BDNF has antidepressant-like effects and enhances hippocampal neurogenesis[228,229], which suggests an important role of this factor in the potentiation of resilience. Chronic SD stress decreases BDNF/TrkB in the PFC, the dentate gyrus (DG), and the CA3 region of the hippocampus, but increases BDNF/TrkB in the NAcc[175,225]. A differential expression of BDNF has been observed in susceptible and resilient mice in function of the brain area studied. Susceptible mice have higher levels of BDNF mRNA in the VTA than resilient and control mice, suggesting that this increase is associated with depressive-like behavior induced by SD[230]. An increase of BDNF-4 has been observed in the PFC of susceptible mice exposed to chronic SD, but the same animals also showed a selective reduction of BDNF-6 transcript in the hippocampus[231]. Conversely, in another study with mice exposed to chronic SD stress, levels of BDNF in the medial PFC and hippocampus were lower in susceptible mice than in control and resilient animals[232]. Finally, several studies support the contribution of hippocampal BDNF expression to resilience to chronic stress[233]. In rodents exposed to SD, activation of hippocampal BDNF/TRKB signaling (by means of branched-chain amino acids, exercise and high protein diets) induces resilience to social avoidance [234,235,236]. In addition, enhancement of BDNF and TRKB levels and signaling has been implicated in the nicotine-induced resilience to the social deficit induced by SD [237].

Hormones of the HPA axis

Stress activates the HPA axis and the release of stress hormones that regulate the individual response to stress. SD stress induces hypercortisolemia and adrenal hypertrophy in susceptible mice, but not in resilient rodents[48,238]. In addition, susceptible mice exhibit reduced glucocorticoid (GR) receptor expression in the hippocampus in comparison to resilient mice, suggesting that up-regulation of GR and enhancement of GR nuclear translocation in the hippocampus play an important role in resilience to chronic SD stress[238]. Susceptible mice show higher plasma corticosterone concentrations 2 h and 48 h after single and chronic SD stress, respectively; and administration of corticosterone *via* drinking water enhances susceptibility while a GR antagonist alleviates the negative consequences of chronic stress[239]. A single dose of ketamine that improved depressive-like behaviors was shown to decrease plasma corticosterone levels and rescue GR expression and nuclear translocation in the hippocampus of susceptible mice[239].

Resilient rats (with proactive behavior in resisting defeat) show decreased efficacy of CRF[82]. Similarly, mice in which CRF is deleted from GABAergic forebrain neurons were found to display a resilient phenotype[240], and PFC mRNA expression of CRF was stronger in susceptible mice than in resilient counterparts[48]. However, another study showed that increasing CRF neuronal activity in a subtype of GABAergic inhibitory interneurons in the medial PFC promoted lasting resilience to SD stress[241,226].

Epigenetic factors

A wide variety of genetic factors - polymorphisms of genes of NPY, CRFR1, catecholamines (COMT, DAT, DAR1, DAR4), serotonin (SERT, 5-HTR1A, 5-HTR3A, 5-

HTR2C), BDNF, among others - have been implicated in resilience (for a review see[4, 242]). Like all aspects of psychological function, resilience results from the interaction between genes and environment. Epigenetic factors are functional modifications to the genome (such as DNA methylation and demethylation, and histone methylation, acetylation, and phosphorylation) that regulate gene expression and phenotype without changing the DNA sequence. Different epigenetic mechanisms have been linked to resilience[243]. For instance, changes in gene expression and chromatin modifications in specific brain regions are associated with resilience to chronic SD stress[70,231,244,245]. In particular, histone methyltransferases are up-regulated in the NAcc of resilient mice, which exhibit low depression-like symptoms after chronic SD [246], while susceptible mice show reduced g9a mRNA levels in the hippocampus, and a reduction of HDAC-5 and DNMT3a mRNA levels in the PFC[231].

HDAC inhibitors may also regulate stress-related behaviors independently of their action on histones, through prevention of glucocorticoid signaling in serotonin pathways. Deletion of HDAC6 in serotonin neurons prevents the electrophysiological and morphological changes induced by chronic SD in these neurons and blocks the expression of social avoidance[247]. In one study, lower acetylated Hsp90 levels, higher GR-Hsp90 association, and enhanced GR translocation were observed in the DRN of vulnerable mice after chronic SD stress, and a HDAC6-selective inhibitor or the serotonin-selective viral overexpression of the acetylation-mimic mutant of Hsp90 in DRN neurons promoted resilience to chronic SD stress[248].

Immune system

Inflammation may underlie individual differences in vulnerability and resilience to chronic SD stress[249,250].

Exposure to SD increases inflammatory markers, but the enhancement of proinflammatory proteins is more pronounced in susceptible rats (with passive coping during defeats and anhedonia) than in active coping rats[236]. In addition, only susceptible rats exhibit elevated levels of inflammatory proteins (IL-1β, TNF-α, GM-CSF) in the LC [251], and higher systemic levels of interleukin-6 (IL-6)[252]. Rats with short-defeat latencies (vulnerable rats) exhibit increased anxiety- and depression-like behaviors, and inflammation in the ventral hippocampus[253]. On the other hand, selective KO of the miR-106b~25 cluster in peripheral leukocytes promotes behavioral resilience to chronic SD stress[254]. Preexisting individual differences in the sensitivity of the peripheral immune system (IL-6) may predict vulnerability or resilience to social stress [250].

Gut microbiota, important activators of inflammatory substances, have emerged as a putative mechanism for promoting stress vulnerability[253]. For example, in one study, mice that were most susceptible to the behavioral effects of chronic SD (reflected by severe social avoidance behaviors) displayed the greatest changes within particular sets of bacteria in the phylum and genus taxonomic ranks[255].

RESILIENCE TO THE EFFECTS OF SOCIAL DEFEAT ON THE REWARDING PROPERTIES OF DRUGS OF ABUSE

There is a well-known link between stress and the development of AUD/SUD, and preclinical studies have shown that early life stress, social rank stress, and SD stress impact on vulnerability and resilience to alcohol, cocaine and other drugs of abuse[14, 15,256]. However, as mentioned previously, there are few works studying resilience to the effects of social stress on the rewarding properties of drugs of abuse. For example, in the search "social defeat, addiction, resilience" in PubMed we identified only 18 papers, and some of these studies did not employ any paradigm of drug reward or addiction. After an exhaustive search and review of the literature we found only 8 papers on resilience to the consequences of repeated or chronic SD for the rewarding effects of cocaine, alcohol or methamphetamine.

In a classic preclinical study of resilience, Krishnan *et al*[70] demonstrated for the first time that, following exposure to chronic SD stress, mice can be classified as susceptible or resilient according to their differential response to stress. Susceptible mice exhibited anhedonia, social avoidance and anxiety-like behavior in the EPM, while resilient mice did not show such symptoms. This study was also pioneering in demonstrating differences between susceptible and resilient mice in sensitivity to the rewarding effects of cocaine. Only susceptible mice showed CPP after conditioning with a low dose of cocaine, while resilient or non-stressed mice did not acquire CPP [70]. Surprisingly, until recently, no other studies have addressed this issue.

In our laboratory, we have studied the influence of IRSD stress on the rewarding properties of cocaine in the CPP paradigm. Exposure to four episodes of SD during late adolescence (on post-natal day (PND) 47-56) subsequently increased the sensitivity of adult mice to a low dose of cocaine. In particular, 1 mg/kg of cocaine induced CPP in defeated mice but not in non-stressed control mice[77]. In a recent study, we evaluated whether some animals were resilient to the effects of IRSD. Overall, exposure to SD decreased all measurements related to the open arms of the EPM, immobility in the TST, social contact in the social interaction test, and grooming in the splash test. IRSD exposure also increased the sensitivity of the mice to the rewarding effects of cocaine, since only defeated animals acquired CPP. However, the potentiation of cocaine CPP was not observed in all the defeated mice, as some of them were resilient to the effects of IRSD on cocaine reward[93]. In the same study we characterized the behavioral profile of vulnerable and resilient mice during defeat episodes and in several behavioral paradigms shortly after SD. Vulnerable mice that showed CPP also exhibited depressive-like behavior, in line with the results of Krishnan *et al*[70]. In comparison with vulnerable mice, resilient mice displayed different behavioral traits, such as less submissive behavior during episodes of defeat, a lower percentage of time in the open arms of an EPM, lower novelty-seeking in the hole board, higher social interaction, greater immobility in the TST, and higher frequency of grooming in the splash test. These results indicate that the behavioral profile of resilient mice is characterized by an active coping response during defeat episodes, a reduced short-term response to SD (lesser reactivity to moderate unavoidable stress, enhanced concern in a potentially dangerous environment and absence of depressive symptoms), and a lack of long-term responses to SD, as evidenced by the absence of cocaine CPP[93].

Two similar studies also showed that control mice do not develop CPP with a low dose of cocaine, while defeated mice did overall develop a preference for the drug-paired compartment[143,162]. Among the defeated animals, two populations could be distinguished - resilient (did not develop preference) and susceptible mice (developed preference) - and they differed in their active or passive behavior during the SD sessions. As the authors stated, "resilient animals showed less flight and submission behaviors than susceptible mice and they presented attack behaviors towards the residents, thereby showing their resistance to being defeated"[162]. Besides passive coping behavior during SD episodes, susceptible mice (which showed cocaine CPP) also displayed social avoidance and higher IL-6 levels in the striatum and hippocampus after the last SD episode[143]. The results of all these studies suggest that an active coping style can protect the individual from the negative consequences of social stress. It is important to note that differences in the responses to cocaine between susceptible and resilient mice were not always observed, since both subgroups of defeated mice showed similar levels of cocaine SA[143].

Rats exposed to repeated SD (five episodes) and social isolation (approximately 12 wk) can also be classified as SD-prone or SD-resilient, based on their affective (depression-like behavior) and cognitive performance. In one study, although SD was shown to increase alcohol SA in both groups, only SD-prone rats displayed heightened motivation for alcohol, persistent alcohol-seeking despite unavailability, resistance to extinction, and increased cue-induced reinstatement of alcohol SA[145]. Similarly, among rats exposed to SD stress, there was a subpopulation in which SD exposure increased anxiety-like behavior and induced escalation of alcohol SA. In comparison with resilient rats, vulnerable rats showed a strong upregulation of vasopressin and oxytocin that correlated positively with the magnitude of the anxiety-like behavior and alcohol SA[146]. These studies suggest that proneness to depression or anxiety enhances vulnerability to AUD, while resilience to mental disorders induced by stress can protect the individual from the development of AUD.

No studies have evaluated resilience to the effects of SD on the rewarding effects of drugs of abuse other than cocaine and alcohol, although one did show that a single SD episode combined with drug priming potentiated the reinstatement of methamphetamine SA (in comparison with priming alone). Interestingly, the defeat latency during the episode of SD correlated with reinstatement values and c-Fos-immunoreactivity in the basolateral amygdala; priming-induced reinstatement and c-Fos were both potentiated in rapidly defeated rats. Conversely, these effects were not observed in rats that were undefeated during the social encounter, but inactivation of the basolateral amygdala induced potentiation of reinstatement, suggesting that this structure mediates resilience against SD stress[144]. The positive correlation between reinstatement and passive coping (high values of reinstatement in animals with lower latency defeat) was reported in another study, although the authors questioned its real meaning, and proposed that active coping behaviors during SD episodes were

associated with the magnitude of reinstatement[257]. In the study in question animals were exposed to SD-predictive cues (discrete environmental stimuli present during the SD stress experience) or not (control group) before reinstatement. Animals exposed to SD-predictive cues exhibited stronger reinstatement of cocaine SA and increased serum corticosterone with respect to the control group. Reinstatement magnitude was positively and significantly correlated with the time spent in a "submissive supine posture", considered a "passive" coping response. However, there was a narrow (though non-significant) correlation between the magnitude of resilience and three behavioral categories indicative of active coping responses; "aggressive allogrooming", "dominant posture", and "retreat". Further studies are needed to determine the real nature of the correlation between the coping strategy of mice during defeat and subsequent vulnerability to reinstatement.

Although scarce, there is research on resilience to the effects of stress on drug reward carried out with other paradigms of social stress. Mice segregated according to whether they are vulnerable or resilient (socially-submissive or socially-dominant mice, respectively) were exposed to CMS for 4 wk; vulnerable, submissive mice displayed a marked increase in cocaine preference after stress, whereas the preference of resilient, dominant mice did not change. In addition, vulnerable mice displayed an increase in the expression of CRF and a reduction in the expression of DA D1 and D2 receptors in the hippocampus[159]. Following exposure to predator odor stress, animals were classified as susceptible or resilient based on EPM behavior and context avoidance; as expected, susceptible animals showed heightened motivation to self-administer cocaine[141]. With the same model, female and male rats were classified according to their stress-reactive behavior (digging and immobility during exposure to the predator odor); no different subgroups could be distinguished in males because all presented the same profile, but female rats were composed of two different populations - high digging/low immobility *vs* low digging/high immobility - and the former showed increased alcohol SA[147].

Early-life adversity consisting of rearing mouse pups in a limited bedding and nesting environment facilitates the escalation of ethanol intake in males at an earlier stage of exposure to alcohol, while females are insensitive to both stress and alcohol. In the study in question, stressed males exposed to alcohol showed reduced open arm exploration in the EPM and increased immobility in the TST compared to alcohol-naïve mice, although they did not differ in grooming response in the splash test, novel object recognition test or corticosterone levels. There were also no differences among control-reared males exposed or not to alcohol. The authors concluded that early stress accelerates the transition from moderate to excessive alcohol drinking and produces anxiety- and depression-like symptoms during alcohol withdrawal[92].

Finally, foot shock stress has been shown to increase two-bottle drinking in some mice, although others show resilience to this effect, displaying higher G-CSF, IL-13, and leptin levels[164]. All these studies suggest that differences in the ability to cope with stressful situations or in the response to stress results in varying tendencies to develop addictive behaviors.

Stress and drug use can lead to common alterations in synaptic plasticity that may contribute to the ability of stress to elicit relapse. For example, disruption of PKC-mediated GluA2 phosphorylation increases vulnerability to both SD-induced enhancement of social avoidance and stress-induced reinstatement of cocaine AA and CPP[258]. Study of resilience to the reinstating effects of SD stress may help to identify therapies that prevent stress-induced relapse. In line with this, Bruchas *et al*[202] demonstrated that SD stress produced reinstatement of cocaine-induced CPP in wild-type mice, but not in mice with a selective deletion of p38α MAPK in DRN serotonergic neurons. The antagonism of DA D3 receptors also prevents the SD-induced reinstatement of cocaine CPP and the increase in corticosterone provoked by SD[259]. Similarly, elimination of Rgs7 (a regulator of G-protein-coupled receptors) in striatal neurons induces a resilient phenotype, since mice do not show SD-induced reinstatement of cocaine CPP and exhibit an anxiolytic- and antidepressant-like profile [163]. Finally, our group has demonstrated that cannabidiol can prevent SD-induced reinstatement of cocaine CPP[154]. Altogether, these studies suggest that resilience to the effects of social stress on relapse to cocaine seeking can be pharmacologically enhanced.

FUTURE RESEARCH DIRECTIONS

An important variable in the development of resilience is sex. Studies on resilience to

stress in animal models have been performed almost exclusively in males, although the prevalence of stress-related disorders clearly differ between males and females [260]. Some studies have shown sex differences in vulnerability and resilience to stress through a lifetime. Prenatal or early stress affects males more than females, inducing problems in social interaction, attention and cognition; conversely, adolescent stress induces more effects in females, increasing the risk of depression, anxiety and PTSD [261]. Thus, research suggests that hormonal activation during puberty, pregnancy or perimenopause highlights the risk associated with stress in females. Furthermore, in comparison with males, female rodents are more resilient to the effects of stress on cognitive processes (for example, in the object recognition task), but are more susceptible to the effects of stress in emotional domains (for example, in the sucrose preference and the FST)[86,87]. In fact, the effects of stress on cognition depend on both sex and the learning task. For example, stress improves the performance of radial arm maze, Morris water maze, Y–maze, non–associative learning, and object placement tasks in females, but impairs it in males[262-264]. Conversely, stress enhances learning of aversive conditioning in males but impairs it in females[265,266]. These data point to the possibility that males and females use different coping strategies in the face of stress[5]. Females are more resilient than males to the impairing effects of chronic unpredictable intermittent restraint on spatial memory, suggesting that chronic stress negatively impairs hippocampal-dependent function in males, but not in females[125]. Sexual differences in the consequences of stress on corticolimbic areas[267], including the brain reward system and the NAcc[202,250], and on the NA LC system[109] have also been reported. In fact, there are sex differences in the effects of ketamine on resilience to chronic stress, as this drug is only effective in males[268]. Research on sex differences in vulnerability and resilience to stress in general and in the field of drug abuse in particular should be a priority of future research. The knowledge obtained by studies with females is critical to the development of effective treatments customized for each sex, which may improve psychological disorders derived from or related with stress, including drug addiction.

Age is another essential variable to be taken into account in the study of resilience to the effects of stress. Adolescents are particularly sensitive to environmental influences, since DA circuitries in the PFC undergo maturational changes at this age. This can render adolescents more vulnerable to the effects of SD. For example, we have observed how SD exposure during adolescence induces a long-term increase in vulnerability to reinstatement[78,155]. A recent study has compared adolescent and adult mice according to their resilience or susceptibility to social avoidance behavior after SD exposure. Although the majority of adolescent mice were resilient, they exhibited risk-taking behavior, alterations in PFC DA connectivity and deficits in inhibitory control when they reached adulthood. Conversely, the majority of adult mice were susceptible (they exhibited social avoidance), but did not show alterations in anxiety-like traits, PFC connectivity or cognition[269]. Chronic SD stress in adolescent mice has a profound impact on the development and plasticity of reward circuity, inducing alterations in the glutamatergic development of the NAcc and mesolimbic DA system [270]. In our laboratory we are now studying the behavioral traits that characterize resilient mice exposed to IRSD stress during early adolescence, with the objective of comparing the results with those of our previous study in adult mice. As maternal experience promotes resilience to the effects of stress on cognition[271], we believe it could be interesting to evaluate how maternity modifies resilience to the effects of SD on drug reward and other behavioral outcomes. The study of stress resilience in the context of aging is very limited[165] but necessary, because the likelihood of the mood and cognitive disorders frequently associated with drug addiction increases in older adults. Thus, future research on resilience must be extended to cover the whole lifespan, with a special focus on critical periods such as prenatal or early life, adolescence, maternity and old age. In addition, it is essential to determine if resilience is a stable trait or changes with time. In this context, a recent study has analyzed the behavior of stressed mice after chronic SD at early and late stages of their lives and has found very dynamic courses of behavior: there are those that are consistently resilient or susceptible over time; those that are susceptible in the short term after stress, but recover with time; and there are animals that are initially resilient but develop vulnerability at a later date[112].

Another area of future research in the field of resilience to the effects of stress on drug addiction is that which explores the behavioral or pharmacological manipulations that increase or promote resilience to the effects of stress on drug reward. Regarding behavioral manipulations, we have observed that mice allowed to perform voluntary physical exercise before exposure to IRSD become resilient to the effects of stress on cocaine-induced CPP (Calpe-López *et al*[93], in preparation). Positive social

conditions, such as paired housing, also increase stress resilience and reverse the potentiation of cocaine reward induced by IRSD, effects that are mediated by oxytocin [161]. In addition, it is essential to improve our understanding of the phenomenon of stress inoculation in order to define the right type and level of stress and the influence of age and sex variables. In recent studies we have tested the effects of different types of stress inoculation, which can modulate the subsequent response of mice to IRSD. In particular, a brief maternal separation (6 h on PND 9) or exposure during adolescence (PND 27) to immobilization, to a single SD or a vicarious defeat experience all induced resilience, and mice did not show cocaine-induced CPP (Calpe-López *et al*[93], in preparation).

Regarding pharmacological manipulations, preclinical studies have shown the effectiveness of several pharmacological treatments in models of resilience to the effects of stress on depressive- and anxiety-like symptoms and in animal models of drug addiction. These studies have highlighted the potential of several drugs that may increase resilience to some of the effects of social stress, including cannabidiol, NMDA antagonists and NOS inhibitors, NPY, galanin, and OX receptor modulators. Thus, it may be interesting to test whether these compounds are a therapeutic target to increase resilience to the effects of stress on drug addiction. Among these compounds, we would highlight cannabidiol, which is effective in reducing PTSD[272] and the effects of cocaine and other drugs of abuse[273,274,275]. A recent study in our laboratory demonstrated that cannabidiol prevents the stress-induced reinstatement of cocaine CPP[154]. Thus, it would be interesting to test the potential of cannabidiol to promote resilience to the effects of stress on the rewarding properties of drugs of abuse. We consider this drug to be a promising pro-resilience compound. Similarly, several studies have demonstrated that the NMDA antagonist ketamine exerts protective effects against the long-term consequences of different kinds of stress in animal models[165,174]. We believe this drug may prevent the long-term effects of stress on drug addiction, as we have demonstrated that another NMDA antagonist, memantine, induces resilience to the effects of social stress on the CPP induced by cocaine in mice[77]. In the same way, the effects of nitric oxide (NO) modulators need to be evaluated, since exposure to stressful events activates NO synthase (NOS), while pharmacological inhibition of NOS reduces depressive and anxious behaviors in animal models[276]. Furthermore, previous studies have demonstrated that NO is also involved in the rewarding effects of drugs of abuse[79,277], and NOS inhibition has been shown to prevent the effects of social stress on the rewarding properties of MDMA in the CPP paradigm[157].

The role of several neuropeptides in the effects of stress in animal models of drug addiction must also be evaluated. As commented on in the previous section, NPY is involved in the regulation of stress responses and plays an important role in emotional behaviors, mediating PTSD and addictive disorders[213-214]. Thus, NPY, as well as galanin[83,84], could induce resilience and prevent the effects of stress on drug addiction. OX play an important role in the response to stress and drugs of abuse [219]. In particular, OX-A activates the HPA axis and induces ACTH and corticosterone release[218]. Furthermore, the antagonism of OX1 receptor blocks the stress-induced reinstatement of cocaine seeking[224,278], and the genetic manipulation of animals to induce a deficiency of OX has been shown to reduce cocaine-seeking after a withdrawal period and responsivity to cocaine-associated cues[22]. Conversely, agonism of the OX2 receptor promotes resilience to the anxiety- and depression-like symptoms induced by SD[224]. Thus, it would be of interest to test whether OX1 antagonists or OX2 agonists increase resilience to the effects of SD in animal models of drug addiction. Finally, p38 MAPKs are key signaling molecules in response to stress, regulation of pro-inflammatory cytokines and drug addiction. For these reasons, p38 MAPK and HDAC6 inhibitors are promising drugs, because they might increase resilience against stress and addiction relapse induced by adverse experiences[160].

It is vital that future research focus on other drugs of abuse, since, with the exception of one study on methamphetamine, cocaine and alcohol are the only drugs to have been evaluated. It is also important to study the relationship between different aspects of resilience (for example, between resilience to the development of depressive symptomatology and resilience against developing drug addiction after stress exposure). Frequently, resilience to a particular effect of stress does not imply resilience to another effect. For example, inhibition of 5-HT synthesis provides resilience against the effects of CMS in the open field, but not in the EPM[103]. Pre-treatment with ketamine before SD protects mice against depressive-like behavior in the FST but does not prevent anxiety-like behavior in the EPM[60]. A recent study showed that susceptibility to SD-induced social avoidance is unrelated to susceptibility to develop a deficit in appetitive, goal-directed motivation after SD; however,

motivational impairments were related to ventral hippocampus hyperactivity, since successful task completion in resilient animals was associated with suppression of ventral hippocampal neural activity[279]. Similarly, rats displaying high cognitive competence in the Y-maze and radial arm maze are also resilient to the negative effects of the FST[280]. Resilience to the effects of SD on cocaine and alcohol reward frequently correlates with the absence of depressive-like symptoms[70,93,143,145].

Finally, translational studies of potential cognitive treatments to increase resilience are essential. For example, cognitive training in humans reduces vulnerability in the face of environmental stress. Similarly, it has been observed that a brief 9-day cognitive training can promote long-term resilience to the CPP induced by cocaine in mice, thus accelerating the extinction of CPP[158]. Coordination between human and animal studies is also required to understand the neural circuit of resilience, and neuroimaging techniques in humans can be combined with classic or more innovative methods in animals.

CONCLUSION

In the fields of medicine and psychology, the concept of resilience has implied a change of paradigm, placing the focus on factors that maintain health and promote wellness. The majority of research on drug addiction aims to identify individual and environmental factors that enhance the vulnerability of a subject to drug addiction. From our point of view, the incorporation of the concept of resilience - a complex, multidimensional construct - will allow scientists to unravel the neurobehavioral traits that confer protection against developing an addictive disorder after exposure to stressful or traumatic events, as well as permitting the underlying neurobiological substrates of resilience to be determined.

Overall, our understanding of the neurobiology of resilience is still at an early stage, but research in the last decade has made leaps and bounds by identifying genetic, epigenetic, molecular, neurochemical, psychological and environmental factors that protect individuals from the neuropsychiatric disorders related to stress[9,281-284]. Currently, resilience is considered an active and dynamic process that can be enhanced to allow individuals to adapt positively to a stressful context that, in other cases, could increase the risk of developing a psychiatric disorder. This concept of resilience has fueled the number of studies focused on specific protective factors and how the neurobiological mechanisms of resilience (HPA axis, GABA, serotonin, glutamate, DA, NA, acetylcholine, endocannabinoids, BDNF-TrkB, OX/hypocretin, NPY, galanin, *etc.*) can be manipulated to increase stress resilience in high-risk individuals and thus prevent the development of psychiatric disorders related to stress[165,219,281,284-286]. However, as stated before, most studies to date have focused on resilience to the development of emotional and anxiety disorders, while those on resilience to addictive disorders are few and far between. The members of our research team are pioneers in the study of resilience to stress in the context of drug addiction. Besides identifying behavioral traits that predict resilience in mice[93], we have highlighted behavioral manipulations (papers in preparation) and pharmacological treatments that increase resilience to the effects of stress in preclinical models of drug addiction. In particular, antagonism of glutamate receptors and inhibition of NOS reverses the effects of IRSD on cocaine and MDMA CPP[77,157,287], while cannabidiol reduces the effects of cocaine[154,273,274] and blocks SD-induced reinstatement of cocaine CPP[154].

Although promising, research on resilience to developing drug addiction after stress in animal models is not devoid of limitations; namely, the difficulty of determining the intensity and duration of exposure to adversity, and the definition of a concrete criterion to consider an animal resilient (absence or reduction of substance use; resistance to developing a SUD or to reinstatement of drug seeking, *etc.*). In addition, the incorporation of females and rodents at different developmental ages is crucial if the realities of resilience are to be fully understood.

From a translational point of view, understanding how an individual develops resilience is of paramount relevance to the design of training programs that increase this ability and promote coping mechanisms, especially in subjects with a maladaptive stress response. There is a well-known link between stress and the development of AUD/SUD, anxiety and depression disorders. Comorbidity between these disorders is frequent and associated with more severe symptoms and poor treatment outcomes. Besides reducing addictive behaviors, resilience training may have positive effects on mental health, reducing vulnerability to the development of anxiety, depressive, and cognitive disorders. Advances in the identification of neurobiological substrates of

resilience will help in the development of pharmacological and psychological interventions for enhancing resilience to adversity and stress.

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2. AIMS and HYPOTHESIS

The background, based on our studies and those conducted in other laboratories, can be summarized as follows:

1. Some individuals are not subject to the negative social, psychological and biological consequences of stress that could otherwise compromise their psychological or physical well-being. These subjects are defined as resilient.
2. Resilience is defined as an integrated process involving many peripheral and central mechanisms that promote an appropriate, non-pathological response to stress.
3. Studies performed to date that characterize the resilient response to social stress in animal models (CSDS) have been based mainly on the effects of stress on mood (depression) and anxiety (PTSD).
4. There are no studies focused on resilience to the increase in the rewarding effects of cocaine produced by intermittent exposure to social defeat stress in animals.
5. Exposure to physical exercise after the last experience of social defeat can prevent potentiation of cocaine reward.

Animals exhibiting a phenotype that is resilient to changes in mood and anxiety following CSDS have been characterized extensively (see reviews by Russo et al., 2012; Krishnan, 2014), but animals that are resilient to the increased reinforcing effects of drugs of abuse after IRSD have not been studied. The critical point in defining resilience is to determine what changes make the animal less likely to develop maladaptive behavioral traits. Importantly, our studies are based on a phenotype (greater sensitivity to the rewarding effects of cocaine) that is assessed long after exposure to IRSD; in

this way, our studies evaluate the long-term resilience response, which has additional translational value.

Our overall objective is to study the changes which characterize mice that are resilient to the increased rewarding effects of cocaine induced by IRSD and to explore how to potentiate resilience.

Firstly, we carried out a behavioral characterization of resilient animals in order to determine behavioral markers that identify animals that will adapt better to situations of stress. Secondly, we explored behavioral and/or environmental strategies that can enhance the expression of a resilient response; to do this, we studied whether exposure to different manipulations (including physical exercise or exposure to slight stressful events) prevents the increase in the rewarding effects of cocaine induced by IRSD.

The resilience to stress has been studied primarily in adult subjects, and results should not be extrapolated to adolescent subjects. Therefore, we decided to focus on the phenomenon of resilience during adolescence. Our group is one of the few to have conducted studies on the effects of social defeat in adolescent animals (an animal model of bullying). We have shown that, despite particularities that differentiate them from adults, adolescents also exhibit an increase in the rewarding effects of cocaine after IRSD. Since adolescence is a critical developmental phase and mice at this age show higher vulnerability to stress, we considered it relevant to perform a specific study of resilience in adolescents.

The specific objectives and hypotheses are described in detail below.

1. *To determine the behavioral traits associated with resilience to the effects of IRSD on cocaine-induced conditioned place preference in mice.* **(Study 1)**

To achieve this **objective**, we exposed late adolescent mice to IRSD or exploration (no stress condition) and evaluated the existence of individual differences in their behavioral response, discriminating between mice that were vulnerable / resilient to the effects of IRSD. In addition, after the last episode of social defeat, we submitted the mice to several behavioral tests (EPM, hole-board, social interaction, splash and tail suspension tests) in order to characterize the behavioral profile of animals that are resilient to the long-term effects of social defeat on cocaine-induced CPP.

Our **hypothesis** was that the behavioral profile of stress-resilient mice in the different behavioral tests would be the same as that of unstressed mice; namely, that vulnerable mice would show CPP with a subthreshold dose of cocaine that does not induce this effect in control and resilient mice. Furthermore, we expected resilient mice to display fewer depressive and anxiogenic symptoms and less novelty-seeking in the behavioral tests.

2. *To evaluate whether Voluntary Wheel Running promotes resilience to the negative consequences of IRSD in mice.* **(Study 2)**

To achieve this **objective**, mice were exposed to voluntary wheel running (VWR) from early adolescence before exposure to IRSD or exploration (no stress condition) in late adolescence (a control group was not exposed to VWR). All the mice then underwent the described previously

behavioral tests a short time after the last defeat and later performed the CPP procedure in adulthood.

Our **hypothesis** was that physical exercise would be an effective tool to prevent the short- and long- term detrimental effects of social stress. Thus, exposure to VWR would promote the development of resilience to the negative effects of subsequent exposure to IRSD, such as the development of anxiety- and depression-like behavior or a potentiation of the rewarding effects of cocaine.

3. *To assess whether a brief maternal separation inoculates against the effects of IRSD on anxiety- and depression-like behavior and cocaine reward in mice.* **(Study 3)**

To achieve this **objective**, we exposed mice to an acute episode of maternal separation (6 h) in early life (PND 9) before exposure to IRSD or exploration (no stress condition) during late adolescence. A control group did not experience maternal separation. All mice then underwent the described previously behavioral tests a short time after the last defeat and later performed the CPP procedure in adulthood.

Our **hypothesis** was that a brief maternal separation in early life would inoculate against the negative effects of subsequent stress and would promote resilience to the effects of IRSD on anxiety-like and depression-like symptoms and potentiation of cocaine reward.

4. *To evaluate whether different acute stressful events can inoculate mice against the effects of IRSD on anxiety- and depression-like behavior and on sensitivity to cocaine reward.* **(Study 4)**

To achieve the **objective** of testing the stress inoculation hypothesis we used different procedures to induce a slight acute stress in early adolescent mice, such as exposure to immobilization stress for 10 min, exposure to an episode of social defeat, or visualization of the social defeat of another mouse (vicarious social defeat). Subsequently, mice were exposed to IRSD or exploration (no stress condition) in late adolescence and then underwent the described previously behavioral tests a short time after the last defeat and later the CPP procedure in adulthood.

Our **hypothesis** was that we would confirm the phenomenon of stress inoculation by exposing subjects to a low level of stress in order to counteract the maladaptive response to future exposure to stress. Thus, we expected that the environmental manipulations performed during early adolescence would be effective in increasing resilience to the effects of IRSD on anxiety, depression or cocaine sensitivity.

5. *To evaluate resilience to the behavioral short-and long-term effects of IRSD in adolescent male mice.* **(Study 5)**

To achieve this **objective,** we first exposed early adolescent mice to IRSD or exploration (no stress condition) and recorded individual differences in their behavioral response, discriminating between mice that were vulnerable and those that were resilient to the effects of IRSD. In addition, after the last episode of social defeat, we performed several behavioral tests

(EPM, hole-board, social interaction, splash and tail suspension tests) in order to characterize the behavioral profile of animals that are resilient to the long-term effects of social defeat on cocaine-induced CPP.

As in Study 1, our **hypothesis** was that the behavioral profile of stress-resilient mice would be similar to that of unstressed mice. In addition, we expected to detect differences between the behavioral profile of resilient mice exposed to IRSD in early versus late adolescence.

3. MATERIAL and METHODS

In this section we describe the Material and Methods employed in **Studies 1 to 5** of the present Doctoral Thesis.

3.1 Experimental animals

Male mice of the C57BL/6 strain were used (Charles River, France). They were delivered to our laboratory on postnatal day (PND) 21 and were housed in groups (4-5 mice per cage) in plastic cages (25×25×14.5 cm). All the mice housed in the same cage underwent the same experimental conditions, and the composition of each cage remained stable throughout each study. To reduce their stress levels in response to experimental manipulations, experimental mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures.

Male mice of the OF1 strain (Charles River, France) were used as aggressive opponents. These animals were individually housed in plastic cages (23×13.5×13 cm) for at least a month before the experiments to induce heightened aggression (Rodríguez-Arias et al., 1998).

All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available ad libitum, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocols were approved by the Ethics Committee of Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045, A1549371980205, 2019-VSC-PEA-056).

3.2 Drugs

In all the studies, C57BL/6 mice were injected intraperitoneally with cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The same physiological saline was also used to dissolve the cocaine. The doses of cocaine (1 or 1.5 mg/kg for mice defeated in late or early adolescence, respectively) were selected on the basis of previous studies (Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

3.3 Animal models and Behavioral tests

The two main animal models used in all the studies were the *Conditioned Place Preference (CPP) and the Intermittent Repeated Social Defeat (IRSD)* paradigms, which were employed to assess the rewarding properties of cocaine and to induce social stress, respectively. Moreover, we performed several behavioral tests including the Elevated Plus Maze (EPM), Hole-Board, Social Interaction, Splash and Tail Suspension Tests in each of the five studies.

3.3.1 Conditioned Place Preference (CPP)

- Materials

Eight "Three Compartment Place Preference with manual doors for mice" from Med Associates Inc. (med-associates.com) were employed in our experiments. These apparatuses consist of identical Plexiglas place conditioning boxes with two equally sized compartments (30.7 x 31.5 x 34.5 cm) separated by a gray central area (13.8 x 31.5 x 34.5 cm). They are fitted

with manually operated guillotine style doors on either side of the neutral gray center compartment for controlling access to both ends of the chamber. The compartments have different colored walls (black vs. white) and distinct floor textures (smooth in the black compartment and rough in the white one). Four infrared light beams in each compartment of the box and six in the central area allow the position of the animal and its crossings from one compartment to the other to be tracked. The equipment is connected to one interphase and is controlled by an IBM PC computer using MONPRE 2Z software (CIBERTEC, SA, Spain) *(See Fig. 4a).*

- Methods

The CPP paradigm evaluates the positive and pleasant properties of stimuli (including the rewarding effects of addictive drugs) (Bardo and Bevins, 2000; Tzschentke, 1998, 2007; Aguilar et al., 2018). In this paradigm, contextual or environmental stimuli acquire secondary appetitive properties (conditioned rewarding effects) when paired with a primary reinforcer (Tzschentke, 1998, 2007). Conditioned reward implies that animals attribute positive incentive value to the cues associated with the primary reinforcer (the drug of abuse), and thus display voluntary responses to obtain access to said cues (Robbins, 1978).

Our protocol, unbiased in terms of initial spontaneous preference, took place during the dark phase, between 10:00 and 14:00 h, and was performed as described below:

a) In the first phase, referred to as Pre-Conditioning (Pre-C), mice were allowed access to both compartments of the apparatus for 15 min (900s) per day on 3 consecutive days. On the last day, the time spent in each

compartment over a 900-s period was recorded. We used a counterbalanced design to assign the mice in each group to the drug- or vehicle-paired compartment. An important requirement of the experimental procedure of CPP is to avoid any preference bias prior to conditioning. Thus, after assigning the compartments, we performed an analysis of variance (ANOVA) with the data of the time spent in each compartment during the Pre-C phase in order to verify the absence of significant differences between the time spent in the compartment paired with the drug and that spent in the compartment paired with vehicle.

b) The second phase (Conditioning) lasted 4 days. In this phase mice underwent 2 pairings per day, on 4 consecutive days. Animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. The order of injections (cocaine or saline) was alternated every day. Confinement was imposed by closing the guillotine door that separates the two compartments. The central area of the apparatus was never accessible during conditioning.

c) During the third phase, known as Post-Conditioning (Post-C, day 8), the guillotine door separating the two compartments was removed and the time spent by the untreated mice in each compartment was recorded during a 900-s observation period. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test versus the Pre-C phase (day 3) is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-

paired compartment, while the opposite indicates that an aversion has developed *(See Fig. 4b)*

Fig.4. a) Apparatus with manual doors for mice used for the Place Preference Conditioning. b) Schematic Cocaine-CPP Procedure

3.3.2 Intermittent Repeated Social Defeat (IRSD)

- Materials

-Experimental animals: We used male mice of the C57BL/6 strain (Charles River, France), which were 21 days of age on arrival at the laboratory (adolescents). They were housed in groups of four in plastic cages (25 x 25 x 14.5 cm).

-Aggressive Opponents: We used male OF1 mice of 42 days of age on their arrival at the laboratory. They were housed singly in plastic cages (23 x 32 x 20 cm) for a month prior to experiments in order to induce aggression (Frick and Gresack, 2003). It should be noted that the home-cage of aggressive opponents was longer because we used a resident–intruder model to induce social defeat.

-Wire mesh barriers, to separate experimental and opponent mice during the first and last 10 min of the social defeat encounters.

-Video camera, computer and computerized program (Raton Time 1.0 software; Fixma SL, Valencia, Spain) to record and analyze the behavior of experimental and opponent mice during the social encounters.

- Methods

-Induction of aggressiveness in the opponents: to ensure that opponent mice exhibited aggressive behaviors, they lived alone for at least one month and were briefly and sporadically confronted with other isolated mice to instigate threat and attack behaviors.

- IRSD: the experimental mouse was defeated in the context of a "intruder–resident" paradigm of aggression based on the fact that an adult male rodent will establish a territory when given sufficient living space. The experimental animal (intruder) was placed in the home cage of the opponent (resident) mouse. As a consequence of isolation and territoriality, the resident showed offensive aggression towards the unfamiliar male mouse that was introduced into its home cage. The intruder showed defensive/submissive behavior in response to the offensive attacks by the resident. In order to minimize physical harm while maintaining stressful effects, the intruder was protected from attack by the resident by a wire mesh barrier during the main part of the encounter and was physically exposed to the resident for only a brief time.

Each episode of social defeat (25 min) consisted of three phases:

a) First phase: The experimental animal was introduced into the home cage of the resident aggressive opponent for 10 min, but the animals were separated by the wire mesh, which protected the intruder from the attack (bites) of the

resident animal. Social interaction and species-typical threats from the aggressive resident (i.e., provocation and instigation) were possible across the mesh (Stoops et al., 2007; Larson and Carroll, 2005; Mateos-García et al., 2015).

b) Second phase: The wire mesh was removed and direct confrontation was allowed for 5 min. We considered the experimental (intruder) to be defeated when it adopted an upright submissive position for 5 s (Ennaceur and Aggleton, 1997; Boissier and Simon, 1962). This posture normally appears after 3–5 attacks by the resident mouse.

c) Third phase: In this last phase, both animals were again separated by the wire mesh for 10 min and the intruder animal was exposed to provocation and threatening behaviors from the resident animal.

Mice were exposed to one episode of social defeat every 72 h, in a total of 4 episodes. Although the experimental animal was usually exposed to a different aggressive animal in each aggressive episode, we always used the same opponent when confronting experimental C57BL/6 mice with OF1 residents in order to reduce the aggressive contacts received by the smaller-sized experimental animal *(See Fig. 5).*

The first and fourth episodes of defeat were video recorded so we could subsequently evaluate the offensive behaviors (threat and attack) of the resident and the defensive/submissive and avoidance/flee behaviors of the experimental animal.

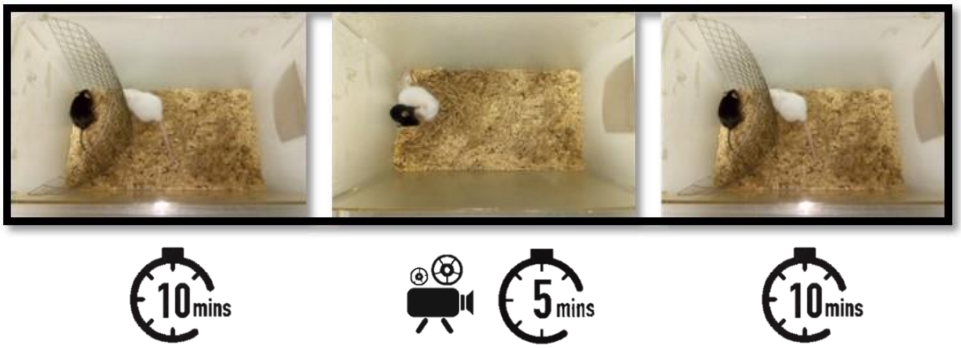


Fig 5. The three different phases of the Social Defeat procedure

-Behavioral analysis of agonistic encounters: The behavioral actions and postures displayed by the mice during the confrontations were video recorded and subsequently analyzed to evaluate the frequencies, durations, latencies, and temporal and sequential patterns of the different behaviors (ethogram). Submissive and fleeing behaviors of the experimental animals and the aggressive behaviors of the opponents were evaluated using a custom-developed program that allows estimation of the time spent engaged in different behaviors (mainly threat, attack, avoidance/flee, and defense/submission) *(See Fig. 6).*

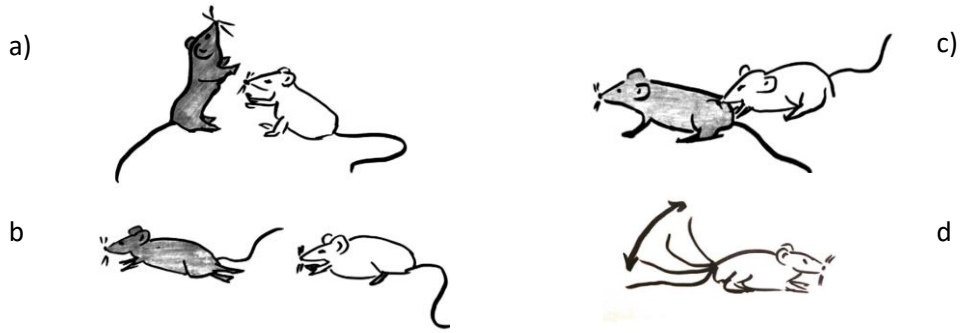


Fig 6. a) Defense/submission and b) Avoidance/flee in grey (intruder). c) Attack and d) Threat in white (aggressive opponent)

-Control group without defeat: We used a control group of mice that did not suffer stress. In this case, the experimental animal was placed in a cage (equal size to the home-cage of the resident) without any other mouse for 25 min, although a wire mesh wall was inserted in the cage on the first and last 10 min to mimic the conditions of stressed mice (Stoops et al., 2007; Takeda et al., 1998) *(See Fig. 7).*

Fig. 7. Exploration condition of Social Defeat

-Short- and long-term behavioral effects of IRSD: we have evaluated the short-term effects of RSD on several behavioral tests, including tests of anxiety- or depression-like symptoms. As we commented before, the experimental animals were exposed to four episodes of social defeat on PND 47, 50, 53, and 56 or on PND 27, 30, 33, and 36 in the case of late and early adolescent mice, respectively. On PND 57-58 (late adolescence) or 37-38 (early adolescence), mice performed the EPM, Hole-Board, Social Interaction, Splash and Tail Suspension Tests. Furthermore, 3 weeks after the last episode of defeat stress, animals underwent the place conditioning procedure with cocaine to evaluate the long-term effects of IRSD on the acquisition of the CPP induced by cocaine.

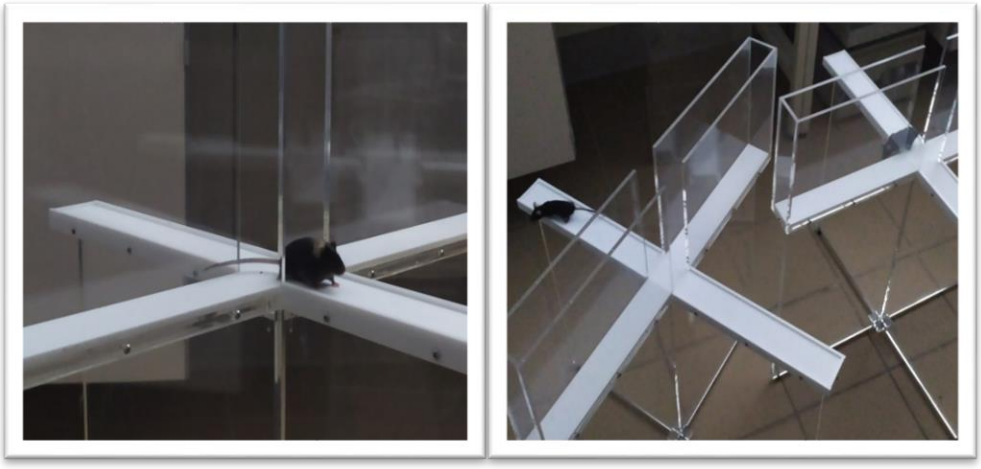
3.3.3 Behavioral Tests

We have evaluated the long-term effects of IRSD on some behaviors, such as anxiety or depression. All tests were conducted on PND 57 and 58, 24 or 48 hours after the last defeat or exploration, except in Study 5, in which they were conducted on PND 37 and 38. During the procedures the animals had no access to food or water. All the apparatuses were carefully cleaned with 70% alcohol after each test.

-Elevated Plus Maze

The effects of IRSD on anxiety were evaluated using the EPM paradigm. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments; therefore, it measures the extent to which rodents avoid high open spaces. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of white Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and the measurements in the closed arms are lower, and vice versa (Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997). Moreover, the total entries into the arms are regarded as locomotor activity

scores (Campos et al., 2013; Valzachi et al., 2013). At the beginning of each trial, subjects were placed on the central platform facing an open arm and were allowed to explore for 5 min. The maze was cleaned with a 7% alcohol swab after each test, and the device remained untouched until completely dry. The behavior of the mice was video recorded and later analyzed by an investigator who was blind to the experimental conditions, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The measures recorded during the test period were frequency of entries and time spent in each section of the apparatus (open arms, closed arms and central platform). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: the latency to first enter the open arms, the time and percentage of time [(open/open + closed) × 100] spent in the open arms, the number and the percentage of open arm entries and total



entries into the arms *(See Fig 8).*

Fig. 8. White Elevated Plus Maze

-Hole-Board Test

The hole-board test evaluates the tendency of rodents to explore a new environment in a free-choice procedure. This test was developed in 1962–1964 by Boissier and Simon (Boissier and Simon 1962; Boissier et al., 1964), and is a simple and useful procedure to assess the response of an animal to an unfamiliar setting (Calabrese, 2008). The exploratory behavior measured in this test is the number of head dips, which represents exploratory tendencies distinct from general locomotor activity. Number of head dips is a useful measure to study the relationship between novelty-seeking and drug abuse (Kliethermes et al., 2007; see Chapter 4 in the Annex). The hole-board consists of a box (28 x 28 x 20.5 cm) with walls made of clear Plexiglas. In the floor of the box there are 16 equidistant holes with a diameter of 2.3 cm. Photocells below the surface of the holes detect the number of times the mouse performs a head dip (Med Associates, CIBERTEC, SA, Spain). A computerized system records the number of times a mouse explores a specific hole and the total frequency of dips performed (Activity Monitor v.7). At the beginning of the test, the mouse is placed in one corner of the hole-board and

allowed to explore it freely for 10 min. The total number of heap dips and the latency to perform the first head dip is recorded *(See Fig. 9).*

Fig.9. Hole-Board apparatus and a computerized system

-*Social Interaction Test*

The social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated Plexiglas cage (10 × 6.5 × 30 cm) was placed in the middle of one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions. The first time (object phase), the perforated Plexiglas cage was empty. After 10 min exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was confined to the perforated cage (to safeguard the experimental mouse from attack) and the experimental mouse was reintroduced into the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the IRSD episodes). In both phases, the time spent in the 8 cm area surrounding the perforated cage -the interaction zone- was registered and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An index of social interaction (ISI) was obtained [time spent in the interaction zone during the social phase/(time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase); Henriques-Alves and Queiroz, 2016]. The ISI is commonly used as the social preference-avoidance index (Krishnan et al., 2007) *(See Fig. 10).*

Fig. 10. Social Interaction Test during the social phase

-Splash Test

The splash test consists of spraying a 10% sucrose solution on the dorsal coat of a mouse placed in a transparent cage (15 × 30 × 20 cm) with regular bedding to stimulate grooming behavior. The behavior of the mice was videotaped for 5 min and later analyzed by an observer who was blind to the treatment received by the animal, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The latency to the first grooming, the time spent engaged in this behavior and its frequency were recorded. An increase in the latency of grooming and a decrease in the time and/or frequency of grooming are interpreted as depressive-like behavior (Smolinsky et al., 2009) *(See Fig. 11).*

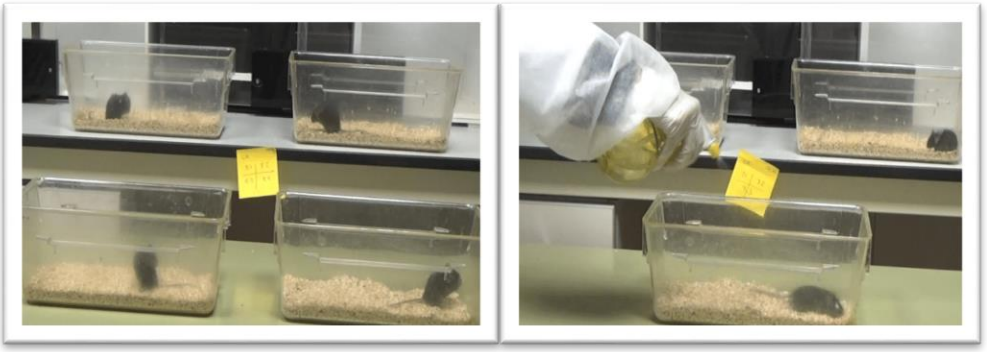


Fig. 11. Splash Test

-Tail Suspension Test

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair (Pollak et al., 2010). It is based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung in an uncontrollable fashion by the tail (Cryan et al., 2005). This has been used as a measure of behavioral depression because, when antidepressant treatments are given prior to the test, the subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle (Pollak et al., 2010). We investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the TST. Following the protocol described by Vaugeois et al. (1997), mice were suspended by the tail, using adhesive tape, from a hook during a 6-min period. The behavior displayed by the mice was video recorded and later analyzed by an observer who was blind to the treatment received by the animal, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameters considered in the

statistical analyses were the total time spent immobile and the latency to show immobility *(See Fig. 12).*

Fig. 12. Tail Suspension Test

In **Studies 2, 3** and **4,** in addition to the CPP and IRSD paradigms and the behavioral tests described before, we carried out specific protocols that are described below.

-Voluntary Wheel Running (Study 2)

We used eight Low-Profile Wireless Running Wheels for Mice (Med Associates Inc.). Each wheel, made entirely of plastic (Overall: 15.24 cm L x 15.5 cm W x 10.16 cm H; Base: 15.24 cm L x 13.72 cm W; Wheel: 15.5 cm W x 3.3 cm H), rotates on a central axis in a horizontal plane, allowing physical activity through natural exercise/spontaneous locomotion (Reguilon et al., 2020). All animals in the exercise condition were distributed in batches of eight and ran individually on the wheel [placed in a plastic cage different to the home cage (23 cm × 32 cm × 20 cm)] under a schedule of 1 hour, three

times per week (Monday, Wednesday and Friday). Control animals were placed in the same plastic cages (different from their own) without any exercise wheel. Each mouse performed a total of 11 VWR sessions *(See Fig.14).*

Fig. 14. Voluntary Wheel Exercise

-Maternal Separation (Study 3)

Newborn mice were separated from their mothers for 6 h (9:00h–15:00h) on PND 9 (following a slight modification of the procedure employed in Llorente-Berzal et al., 2013). We selected PND 9 for MS because this day marks the end of the neonatal period (PND 3-9) and the initiation of the postnatal transition (PND 9-15) (Fox, 1965). In addition, by this stage (PND 9) mice show full retention 24 h after learning (Alleva and D' Udine, 1987).

During separation, the mother was removed and placed in another cage (23 cm × 32 cm × 20 cm) with access to food and water, while the pups remained in their home box. No specific procedure was used to keep the litter warm during this period, as the room temperature in the laboratory was maintained at 21°C and pups have a thick (almost complete) fur by PND 9, which allows thermoregulation. After 6 h, the mother was placed once again with her litter. Weaning was carried out on PND 21, during which the mice were separated by sex (Calpe-López et al., 2022b) *(See Fig. 13).*

Fig. 13. Pups during Maternal Separation

-Stress immunization protocols (Study 4)

The following protocols were carried out in early adolescence (PND 27).

Immobilization

To evaluate the effects of acute immobilization stress, the animals were submitted to restraint for 15 min on PND 27. Restraint is a powerful stressor widely used in many studies (Patel et al. 2005; for a review see Lu et al. 2003). Restraint was induced as follows: when mice spontaneously entered a cylindrical glass tube (4 cm in diameter and 10 cm in length, with holes of 0.5 cm in diameter to permit respiration), two test tubes of 0.5 cm in diameter were carefully introduced underneath the animal to reduce the size of the diameter of the tube to 3 cm so that it was impossible for the animal to turn (Ribeiro Do Couto et al., 2006) *(See Fig. 15).*

Fig. 15. Acute immobilization

Acute Social Defeat

In this paradigm the experimental animal suffered a short experience of social defeat during a 10 min period on PND 27 (early adolescence) to inoculate against stress. We used a neutral transparent plastic cage for the agonistic encounters (23 x 13.5 x 13 cm), different from the home cages of the experimental and aggressive animals. First, both animals were placed in this cage but were separated by a transparent plastic barrier for 1 min. The barrier was then removed and physical interaction was allowed for 10 min. In response to the aggressive behaviors of the opponent (an isolated OF1 mouse), the experimental animal (which was not housed in isolation and did not have fighting experience) exhibited avoidance/flee and defensive/submissive behaviors. The criteria used to define an animal as defeated was a specific posture, characterized by an upright position, limp forepaws, upwardly angled head, and retracted ears *(See Fig 6a)*.

For specifics regarding Materials (experimental animals, housing conditions, induction of aggressiveness in the opponents, etc.) and Methods, we refer the reader to the standardized protocol described above for *IRSD*.

Vicarious Social Defeat (VSD) Stress

- Materials

-We refer the reader to the IRSD materials for information on housing conditions, animals used and the induction of aggression in opponents.

-Cage for the agonistic encounters: We used a neutral transparent plastic cage (29 x 60 x 35 cm) different from the animals' home cage.

-Wire mesh barriers: We used two meshes made of a metal grid (9 x 60 x 35 cm), each of them consisting of six compartments (9 x 10 cm), to separate the defeated and opponent mice during the social defeat encounters.

- Methods

- Induction of VSD in the experimental mouse: An agonistic encounter between the defeated mouse and the aggressive opponent took place through a central corridor in a neutral transparent plastic cage *(See Fig. 16),* so that the rest of the experimental mice could smell and observe the defeat through the metal grid.

First, the experimental animals were placed in the wire-mesh-separated compartments (six on each side of the plastic cage). An OF1 aggressive mouse was then placed in the intermediate space so it could freely explore the corridor for 3 minutes. Immediately, a conspecific c57 male mouse was introduced into the central corridor and an agonistic encounter took place for 5 minutes, while the experimental mice received a vicarious experience (i.e., visual, olfactory, auditory) of the physical bout *(See Fig. 16).*

- Control group without defeat: We used a control group of mice that did not suffer vicarious stress. In this case, the experimental animals were placed in the wire mesh compartments (six on each side) of the neutral plastic cage and remained there for 8 min without any agonistic encounter visualization.

- Effects of a vicariously observed stress condition: In this paradigm of social defeat, the experimental animals visualize a short experience of social defeat between a conspecific-defeated mouse and an aggressive-opponent mouse on PND 27.

The VSD is a novel paradigm capable of inducing emotional stress by avoiding physical stress/confrontation in mice (Sial et al., 2016). In this paradigm, male mice exhibit depressive-like behaviors after witnessing the defeat of a same-sex conspecific, which resembles the behavioral profile of physically stressed mice (Iñiguez et al., 2014; Iñiguez et al., 2018; Hodes et al., 2014).

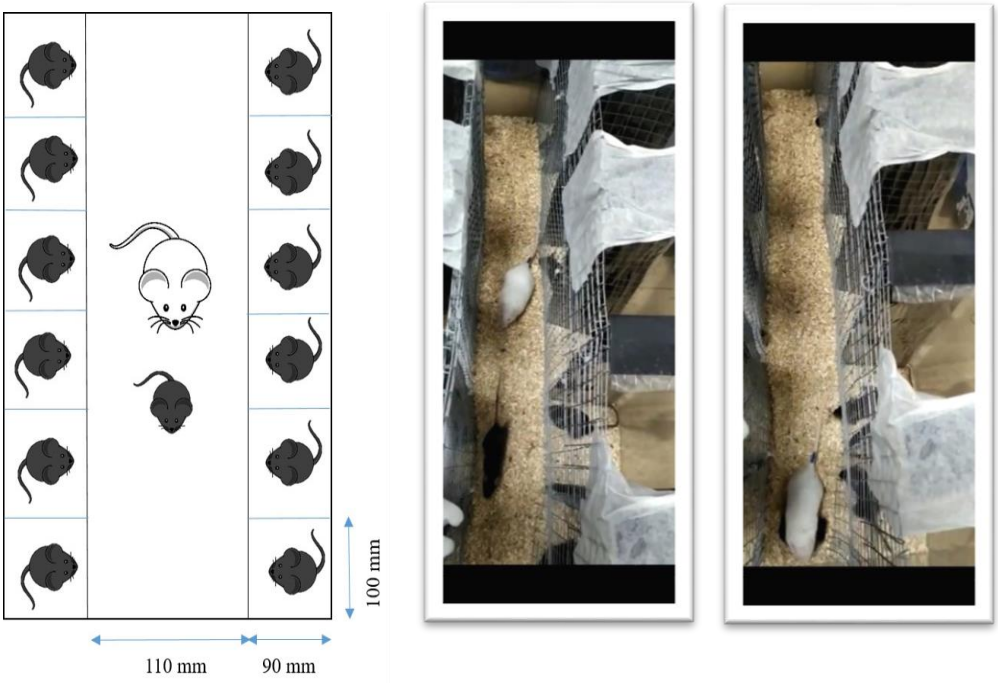


Fig. 16. Vicarious Social Defeat

4. RESULTS

The main results obtained in the different experimental studies performed are described below and summarized in Tables 1, 2 and 3. The reader can find the complete description of results of each study in the Annex with the published, under review or in preparation papers.

Experimental Study 1:

The results show that exposure to IRSD induced anxiety since defeated mice displayed diminished measures in the open arms in the EPM (time spent, number of entries, percentage of time spent and percentage of entries) in comparison to controls. In addition, IRSD reduced social interaction, immobility in the TST and grooming in the Splash Test. Exposure to IRSD also increased the sensitivity of mice to the conditioned reinforcing effects of cocaine, as only the defeated group of animals showed CPP after conditioning with 1 mg/kg cocaine, a dose that was ineffective in the non-stressed control group. However, in the defeated group of animals, two subgroups were distinguished: one vulnerable to the long-term effects of defeat on cocaine-induced reward (which developed CPP), and another resilient subgroup that behaved like the control group (they did not show CPP). Several behavioral traits were related to resilience to the enhancing effect of RSD on cocaine CPP. Resilient mice showed less submission during defeat episodes, a lower percentage of time in the open arms in the EPM, lower novelty-seeking, higher social interaction, greater immobility in the TST, and higher frequency of grooming.

These results suggest that the behavioral profile of defeated mice is characterized by an active coping response during defeat episodes (less

submissiveness), greater concern about potential dangers in unfamiliar environments (EPM and hole-board), less reactivity in a moderate stress situation (TST) and fewer depressive-like symptoms (splash test) after stress. Thus, the results of this first study show that several individual traits contribute to a subject's resilience to the negative consequences of social stress (social interaction deficit, anhedonia and increased sensitivity to drugs).

Experimental Study 2:

The main results of this second study are that exposure to physical exercise (voluntary wheel running, VWR) during early adolescence prevented the negative consequences of social stress in the EPM, splash test and cocaine CPP; the group under stress that had performed physical exercise (VWR+IRSD) did not show anxiety- or depression-like symptomatology or a potentiation of the conditioned rewarding effects of cocaine like that observed in the group of defeated animals without previous physical exercise (CONTROL+IRSD). However, exposure to physical activity did not prevent the social interaction deficit elicited by ISRD. Thus, our findings support the idea that physical activity promotes resilience to some negative effects of subsequent social stress and represents an excellent tool for the prevention of drug abuse or other stress-related mental disorders.

Experimental Study 3:

The results of this study show that, regardless of whether maternal separation (MS) had occurred or not, lower measures of open arms in the elevated plus maze, novelty-seeking and social interaction were observed in

mice that had experienced defeat (MS+IRSD and CONTROL+IRSD). However, increased grooming latency and cocaine-induced CPP acquisition were only observed in mice exposed to defeat (CONTROL+IRSD). These results indicate that an episode of MS prevents some effects of IRSD exposure in late adolescence, such as the development of depressive-like symptomatology and the potentiation of cocaine-induced CPP. However, MS did not modify social avoidance behavior or social defeat-induced anxiety since MS did not prevent the effects of IRSD in the EPM; mice exposed to MS + IRSD exhibited a similar profile to mice exposed only to defeat (CONTROL + IRSD). Indeed, mice exposed to MS + IRSD showed a lower percentage of entries in the open arms of the EPM and a reduction in the distance travelled in the EPM with respect to non-stressed mice (CONTROL + EXPL group), neither of which was the case among mice exposed to IRSD or MS alone. On the other hand, mice exposed to MS + IRSD performed a greater number of stretch-attend postures than mice in the CONTROL + IRSD and MS + EXPL groups, and the MS + IRSD group was the only one that did not differ from control mice (CONTROL + EXPL group), suggesting the development of resilience to this effect of IRSD.

Thus, these results suggest that inoculation against stress early in life through a brief episode of MS enhances later resilience to some of the negative effects of IRSD stress by preventing the development of depressive-like behaviors produced by defeat in late adolescence and the long-term increase in their sensitivity to the rewarding effects of cocaine in adulthood.

Experimental Study 4:

The results of this study give support to the hypothesis that stress inoculation promotes resilience to the effects of IRSD on cocaine reward. Brief acute exposure to immobilization (IMM, physical stress), an acute social defeat (SD) in an agonistic encounter with an aggressive opponent (social stress), or an acute experience of vicarious social defeat (VSD) stress through visualization of the social defeat of another mouse (emotional stress) in early adolescence conferred resilience to the long-term effects of exposure to IRSD on the rewarding properties of cocaine. Inoculated mice subsequently exposed to IRSD in late adolescence did not develop cocaine-induced CPP in adulthood, while non-inoculated mice exposed to IRSD showed enhanced sensitivity to cocaine and effectively acquired CPP. Stress inoculation also prevented some short-term effects of IRSD, although results were in function of the stressor experienced by mice in early adolescence. In particular, exposure to acute social defeat prevented the social avoidance observed in mice exposed only to IRSD, while exposure to acute immobilization prevented anxiety-like effects of IRSD in the EPM (in all measures in the open arms), and vicarious social defeat also prevented the increased latency to enter the open arms of the EPM observed among mice exposed to IRSD alone. It is important to note that stressful stimuli employed to induce stress inoculation also induced long-term effects in mice that had not suffered stress in late adolescence, including a reduction of grooming in the splash test (acute social defeat and immobilization), a decrease of immobility in the TST (vicarious social defeat and immobilization), an increase in novelty-seeking, social interaction and cocaine CPP (immobilization) and an increase in the number of entries and time spent in the open arms of the EPM (vicarious social defeat).

Experimental Study 5:

The results of this study revealed a deficit in social interaction, a decrease in the frequency of grooming in the splash test and an increase in the rewarding effects of cocaine in animals subjected to ISRD in early adolescence compared to non-stressed animals. However, IRSD produced no significant effects in the EPM, hole-board or tail suspension test. Furthermore, as occurred in animals exposed to stress during late adolescence, we were able to distinguish two subgroups among the animals exposed to ISRD in early adolescence: one that was vulnerable and one that was resilient to the effects of stress on cocaine-induced CPP. With respect to the behavioral characteristics of the resilient animals, our findings highlight less submissiveness during defeats, an absence of depressive-like symptomatology (in the social interaction test and in the splash test) and an enhanced concern about potential dangers (lower percentage of time in the open arms of the EPM).

	STUDY 1		STUDY 5	
	Vulnerable adult mice	Resilient adult mice	Vulnerable adolescent mice	Resilient adolescent mice
Cocaine reward (CPP)	↑	=	↑	=
Submission (Agonistic encounters)	↑	↓	↑	↓
Open arms measures (EPM)	=	↓	=	↓
Novelty-seeking (Hole-board)	=	↓	n.d.	n.d.
Social interaction (SI)	↓	=	↓	=
Grooming (Splash test)	↓	=	↓	=
Immobility (TST)	↓	=	n.d.	n.d.

Table 1. Summary table of results (Study 1 and 5). = mice showed similar values to mice without exposure to social defeat. ↓ mice showed lower values than mice without exposure to social defeat; ↑ mice showed higher values than mice without exposure to social defeat; n.d. the behavioral profile of mice in this test did not differ between resilient and vulnerable mice.

Study 1. After IRSD exposure in late adolescence, vulnerable mice developed CPP with a low dose of cocaine that did not induce CPP in controls and resilient defeated mice. Reduced defensive/submissive behavior during episodes of defeat, avoidance of the open arms of the elevated plus maze (EPM) and lower novelty-seeking were behavioral traits that predicted resilience to the effects of IRSD on cocaine CPP. Resilient defeated mice behaved similar to controls in the social interaction (SI) test, splash test and tail suspension test (TST). Conversely, increased defensive/submissive behavior during episodes of defeat, hyperreactivity in a stressful situation (TST) and depressive-like behaviors (social avoidance and anhedonia after IRSD) were behavioral traits predictive of vulnerability to the effects of IRSD on cocaine CPP.

Study 5. With respect to the behavioral characteristics of the animals that proved resilient to the effects of IRSD in early adolescence, we highlight less submissive behavior during defeats, an absence of depressive-like symptomatology (in the social interaction test and in the splash test) and a higher concern for potential dangers (lower percentage of time in the open arms of the EPM).

	STUDY 1	STUDY 2			STUDY 3		
	Resilient mice	Control + IRSD	VWR+ Expl	VWR+ IRSD	Control + IRSD	MS+ Expl	MS+ IRSD
Cocaine reward (CPP)	=	↑	=	=	↑	=	=
Open arms measures (EPM)	↓	↓	↓	=	↓	=	↓
Novelty-seeking (HB)	↓	↓	=	↓	↓	=	↓
Social interaction (SI)	=	↓	=	=	↓	=	↓
Grooming (Splash test)	=	↓	=	=	↓	=	=
Immobility (TST)	=	↓	=	↓	n.a.	n.a.	n.a.

Table 2. Summary table of results from Studies 2 and 3 and comparison with resilient mice (Study 1). = mice showed similar values to mice without exposure to stress (the respective control group); ↓ mice showed lower values than mice without exposure to stress; ↑ mice showed higher values than mice without exposure to social defeat; n.a., the behavior of mice was not assessed in this test.

Study 2. Behavioral profile of mice exposed to Voluntary Wheel Running (VWR) + IRSD. Defeated mice exposed to VWR showed a similar behavior to that of resilient mice from Study 1 in the CPP paradigm, hole-board, social interaction and splash tests, but not in the EPM or TST. These results indicate that exposure to VWR during adolescence promotes resilience to most of the subsequent negative effects of IRSD on late adolescence.

Study 3. Behavioral profile of mice exposed to Maternal Separation (MS) + IRSD. Defeated mice exposed to MS showed a similar behavior to that of resilient mice from Study 1 in the CPP paradigm, EPM, hole-board, and splash tests, but not in the social interaction test. These results indicate that exposure to MS during adolescence promotes resilience to most of the subsequent negative effects of IRSD on late adolescence.

	STUDY 1	STUDY 4					
	Resilient adult mice	Control+ IRSD	IMM+ Expl	IMM+ IRSD	ASD+ Expl	ASD+ ISRD	VSD+ Expl	VSD+ IRSD
Cocaine reward (CPP)	=	↑	↑	=	=	=	=	=
Open arms measures (EPM)	↓	↓	=	=	=	↓	=	↓
Novelty-seeking (HB)	↓	↓	↑	↑	=	=	=	↓
Social interaction (SI)	=	↓	↑	=	=	=	=	↓
Grooming (Splash test)	=	↓	↓	↓	↓	↓	↓	↓
Immobility (TST)	=	↓	↓	↓	=	=	↓	=

Table 3. Summary table of results from Study 4 and comparison with resilient mice (Study 1). = mice showed similar values to mice without exposure to stress (the CONTROL+EXPL group); ↓ mice showed lower values than mice without exposure to stress; ↑ mice showed higher values than mice without exposure to social defeat.

Study 4. Behavioral profile of mice exposed to different protocols of stress inoculation: immobilization (IMM), acute social defeat (ASD) or vicarious social defeat (VSD) in early adolescence. Defeated mice exposed to IMM showed a similar behavior to that of resilient mice from Study 1 in the CPP paradigm and social interaction test, but not in the EPM, hole-board, and splash tests. Defeated mice exposed to an ASD showed a similar behavior to that of resilient mice from Study 1 in the CPP paradigm, EPM, social interaction test and TST, but not in the hole-board or splash test. Finally, defeated mice exposed to VSD showed a similar behavior to that of resilient mice from Study 1 in the CPP paradigm, EPM, hole-board and TST, but not in the social interaction or splash tests. These results indicate that exposure to each of the stressful episode during adolescence promotes resilience to the negative long-term effects of IRSD on cocaine reward.

5. GENERAL DISCUSSION and CONCLUSIONS

The majority of research on drug addiction aims to identify the individual and environmental factors that increase a subject's likelihood of becoming addicted. The concept of resilience - a complex, multidimensional construct – refers to the neurobehavioral features that confer protection against developing an addictive disorder after exposure to stressful events, as well as the neurobiological substrates underlying resilience.

In our laboratory, we have used a protocol of defeat consisting of intermittent exposure of mice to an episode of defeat that is repeated four times, every 72 h. With this protocol of intermittent defeats, we have seen how mice exposed to IRSD exhibit anxiety- and depression-like behavior, altered social interaction and learning impairments (Calpe-López et al., 2020; García-Pardo et al., 2017), as well as enhanced sensitivity to drugs of abuse (García-Pardo et al., 2015, 2016, 2019; Calpe-López et al., 2020). However, in the same way that most humans exposed to stress do not develop mental disorders, we have demonstrated that chronically defeated rodents respond to stress differently; some develop anxiety-, depression and addiction-like symptoms, whereas others remain resilient to these effects of stress (Calpe-López et al., 2022a; Wang et al., 2021).

Thus, the main objective of the present thesis was the characterization and potentiation of resilience to the short- and long-term behavioral effects of IRSD exposure in early and late adolescence. Our research regarding resilience to the effects of IRSD on the rewarding properties of cocaine is an important contribution to the field for two reasons. First, most other studies have focused on resilience to depression- or anxiety-like behavior, while resilience against developing drug use disorders after stress has been studied very little. Second, we have evaluated resilience against the effects of IRSD

in late adolescence on cocaine reward in the long term, in adulthood. Thus, our research is pioneering in that it identifies mice that are resilient to the potentiation of cocaine CPP induced by IRSD and characterizes their behavioral profile during social defeat episodes and in a battery of behavioral tests after IRSD (**Experimental Study 1, Calpe-López et al., 2020**). We have also identified and characterized mice which are resilient to the effects of IRSD in early adolescence (an animal model of bulling), following the same experimental protocol (**Experimental Study 5, Calpe-López et al., under review**). The objective in this case was to verify whether the behavioral profile that was predictive of resilience in mice exposed to IRSD in late adolescence (described in the first study) was also applicable to animals exposed to DSRI during early adolescence. It is important to note that early adolescent mice experience social defeat less intensely than their older counterparts. The development of depression-like symptoms (deficit of social interaction and decrease in grooming in the splash test) and an enhanced sensitivity to the conditioned rewarding effects of cocaine in adulthood was observed irrespective of the age the mice were when they were exposed to IRSD. However, while mice exposed to IRSD in late adolescence displayed anxiety-like behavior in the EPM, elevated stress responsivity (reduced immobility) in the TST, and a decrease in novelty-seeking in the hole-board, these effects were not observed among mice exposed to IRSD during early adolescence. Regarding the behavioral traits that predict resilience to the effects of IRSD on the development of cocaine CPP, we observed that the maintenance of an active coping strategy during episodes of social defeat (reduced submissive behavior), the absence of social avoidance, unaltered levels of grooming and a lower percentage of time spent in the open arms of the EPM were observed in resilient mice exposed to IRSD in early or late

adolescence. However, the behavioral profile of mice in the hole-board or tail suspension tests was associated with resilience to the long-term effects of IRSD only in mice exposed to defeat in late adolescence.

The other main objective of our work has been the potentiation of resilience through environmental interventions. We have demonstrated the development of mechanisms of recovery from stress through the protective effects of physical exercise. Exposure to voluntary wheel running (VWR) during adolescence enhanced the resilience of defeated mice and protected them against several negative consequences of stress, such as anxiety- or depression-like behavior (social avoidance and anhedonia) and the potentiation of the rewarding effects of cocaine (**Experimental Study 2**). In addition, we have demonstrated that a brief period of maternal separation (MS) prevents the short-term depression-like effects of IRSD and its long-term effects on the rewarding properties of cocaine. Mice exposed to an episode of MS in early life and to repeated experiences of defeat in late adolescence behave in the same way as non-stressed mice and do not display anhedonia or acquire CPP after conditioning with a low dose of cocaine. However, our MS protocol did not prevent other effects of IRSD, such as anxiety-like behavior, a reduction in novelty-seeking, or a deficit in social interaction (**Experimental Study 3**). Finally, we can affirm the hypothesis of stress inoculation, since exposure to different protocols of inoculation against stress in early adolescence (acute immobilization, acute social defeat or acute vicarious social defeat) promoted resilience to the long-term effects of IRSD on cocaine reward (**Experimental Study 4**).

From a translational point of view, our results support the real-world observation that not all individuals exposed to social stress subsequently suffer mental and/or addictive disorders. According to our results, and to evidence obtained in humans, an active coping strategy (Feder et al., 2009) and a search for social support (Wu et al., 2013) should be encouraged. In addition, it is necessary to decrease reactivity to stressful events and increase awareness of dangers, as well as to promote the self-control function and sense of safety. These changes can be achieved by means of problem-solving tasks, relaxation training and cognitive restructuration (Thompson et al., 2018). Our results also suggest that behavioral interventions that increase the pro-active response of adolescents exposed to bullying can enhance their resilience to the negative consequences of this stressful experience and prevent the development of depressive and addictive disorders. Our results endorse the idea that exercise can prevent the development of stress-related disorders. Physical activity during adolescence is an excellent tool to improve resilience to the negative effects of subsequent social stress on the vulnerability of an individual to mental and addictive disorders later in life. In addition, inoculation of stress in early life or adolescence can increase subsequent resilience to some of the negative effects of social stress, including the enhancement of sensitivity to the rewarding properties of cocaine in adulthood. Nevertheless, it is necessary to carry out further research to determine other protective factors that promote the development of resilience to stress during childhood or early adolescence.

Although promising, research on resilience to the development of drug addiction following stress in animal models is not without limitations; namely, the difficulty in determining the intensity and duration of exposure to adversity and specific criteria for considering an animal to be resilient

(absence or reduction of substance consumption, sensitivity to drug reward, reinstatement of drug-seeking, etc.). In addition, the incorporation of females and rodents at different developmental ages into this field of research is crucial if we are to fully understand the realities of resilience, since there are likely to be some sex and age differences in behavioral markers of resilience.

The results of our research show that there are certain neurobehavioral characteristics that predict resilience to social stress and that it is possible to promote resilience to the short- and long-term negative effects of stress, such as the development of anxiety, depression or drug use disorders. Characterization of resilient animals is the first step in developing behavioral and/or pharmacological strategies that promote the emergence of a resilient response to the negative effects of stress. From a translational point of view, understanding how resilience develops is vital for designing training programs that enhance this capacity and promote coping mechanisms, especially in subjects that are more vulnerable to stress. In addition to reducing addictive behaviors, resilience training can have positive effects on mental health, reducing vulnerability to the development of anxiety, and depressive and cognitive disorders. Advances in the identification of the neurobiological substrates of resilience will no doubt aid the development of pharmacological and psychological interventions to improve resilience to adversity and stress.

Research on resilience can contribute to the development of new approaches to avoid stress-related mental disorders and new therapeutic strategies to treat people at risk of developing a drug use disorder after stressful experiences.

The following is a summary of the main conclusions of each of the studies included in the present Thesis:

Experimental Study 1

- We have demonstrated that the IRSD paradigm is useful to assess how chronic social stress influences vulnerability or resilience to the development of mental disorders, including depression, anxiety and CUD in mice.
- In the short term following exposure to IRSD in late adolescence mice show anxiety-like symptoms in the EPM and lower levels of social interaction, immobility in the TST and grooming in the splash test in comparison to non-stressed mice.
- In the long term after IRSD exposure in late adolescence, mice exhibit a potentiation of the rewarding effects of cocaine; in adulthood they acquire CPP with a dose of cocaine that is ineffective in non-stressed mice.
- Among defeated mice it is possible to distinguish subgroups: a subgroup of vulnerable mice that effectively acquire cocaine CPP, and another subgroup of defeated mice that are resilient to the effects of IRSD on cocaine reward, that behave similar to non-stressed mice in adulthood; i.e. resilient mice do not develop cocaine CPP.
- Resilience to the enhancing effects of IRSD on sensitivity to cocaine reward is associated with a distinctive behavioral profile (in comparison to vulnerable mice), both in social defeat episodes and in tests performed short term after IRSD.

- Resilient mice are characterized by less submission during defeat episodes, less interest in the open arms of the EPM, lower novelty-seeking in the hole-board test, less reactivity in the TST, and an absence of IRSD-induced deficits such as social avoidance and anhedonia (in the social interaction and splash tests, respectively)**.**
- Thus, some mice are resilient to both the depression-like effects (deficit of social interaction and anhedonia) and the enhanced cocaine sensitivity induced by IRSD. Several individual traits, including an active coping response and avoidance of potential dangers in unknown environments, and reduced acute stress reactivity, contribute to a subject's resilience to the negative consequences of social stress.

Experimental Study 2

- We have confirmed that mice exposed to IRSD in late adolescence show anxiety-like symptoms in the EPM and lower levels of novelty-seeking, social interaction, immobility in the TST and grooming in the splash test in comparison to non-stressed mice. In addition, defeated mice acquire cocaine CPP in the adulthood.
- We have demonstrated that exposure to physical activity through voluntary wheel running (VWR) during adolescence enhances the resilience of defeated mice and protects them against some negative consequences of stress. Mice exposed to both VWR and IRSD do not show the anxiogenic- or depression-like behavior (social avoidance

and anhedonia) or the potentiation of the rewarding effects of cocaine observed in mice exposed only to IRSD.

- VWR during adolescence does not prevent the reduction of novelty-seeking and of immobility in the TST induced by IRSD.
- VWR during adolescence in non-stressed mice induced anxiety-like symptoms in the EPM; thus, this procedure may promote future resilience in defeated mice through a process of stress inoculation.

Experimental Study 3

- We confirmed once again that mice exposed to IRSD in late adolescence show anxiety-like behavior and lower levels of novelty-seeking, social interaction and grooming, and acquire cocaine CPP.
- An acute brief maternal separation (MS) in early life does not induce short-term effects in the EPM, hole-board, social interaction or splash tests, and nor does it induce long-term effects on cocaine CPP.
- We have demonstrated that an episode of MS in early life prevents some effects of subsequent IRSD exposure in late adolescence, including increased latency to grooming behavior in the splash test and potentiation of cocaine-induced CPP.
- MS does not prevent most of the short-term effects of IRSD, including social avoidance, reduction of novelty-seeking and anxiety-like behavior.
- Thus, a brief MS of pups is a slightly stressful event that can inoculate mainly against the long-term effects of subsequent exposure to IRSD

and prevent the enhanced sensitivity of defeated mice to the rewarding effects of cocaine.

Experimental Study 4 (in preparation)

- Exposure to three different protocols of inoculation to stress in early adolescence (acute immobilization, acute social defeat or acute vicarious social defeat) promotes resilience to the long-term effects of IRSD on cocaine reward. Mice exposed to IRSD in late adolescence develop cocaine CPP, but defeated mice previously exposed to these stressful events do not develop CPP. These results support the hypothesis of stress inoculation.
- Exposure to an acute episode of immobilization in early adolescence also prevents some of the effects of IRSD exposure in late adolescence (such as anxiety-like behavior, the reduction of novelty-seeking and social avoidance), but other effects of IRSD remain unaltered (such as a decrease in grooming behavior and immobility in the TST).
- Exposure to an acute episode of immobilization in early adolescence induces by itself (without IRSD) behavioral effects in late adolescent and adult mice. Thirty days after exposure to immobilization, mice without additional stress exposure show an increase in novelty-seeking behavior and social interaction but a reduction of grooming and immobility in the TST. Furthermore, mice exposed only to

immobilization in early adolescence acquire cocaine CPP in adulthood.

- Exposure to an acute episode of social defeat in early adolescence also prevents some effects of IRSD exposure in late adolescence (such as the reduction of novelty-seeking, social avoidance and immobility in the TST) but other effects of IRSD remain unaltered (such as anxiety-like behavior and a decrease in grooming behavior).
- Exposure to an acute social defeat in early adolescence induces by itself (without additional stress exposure) a reduction of grooming behavior thirty days after the episode of defeat.
- Exposure to an acute episode of vicarious social defeat in early adolescence only prevents the decrease in immobility in the TST induced by IRSD exposure in late adolescence, while the other short-term effects of IRSD (anxiety-like behavior, reduction of novelty-seeking, social avoidance and decrease in grooming behavior) remain unaltered.
- Exposure to acute vicarious social defeat in early adolescence induces by itself (without additional stress exposure) a reduction of grooming behavior and immobility in the TST thirty days after the episode of vicarious defeat.

Experimental Study 5 (under review)

- We demonstrate that IRSD exposure during early adolescence induces depressive-like behavior (social avoidance and anhedonia in the social interaction and splash tests, respectively). In addition, IRSD in early

adolescence increases the sensitivity of mice to the rewarding effects of cocaine in adulthood.

- Early adolescent mice experience social defeats less intensely than their older counterparts, since mice exposed to IRSD in early adolescence do not show anxiety-like behavior in the EPM or decreased novelty-seeking and immobility in the TST (effects observed in mice exposed to IRSD in late adolescence).
- The IRSD paradigm is useful to assess the influence of chronic social stress on vulnerability or resilience to develop depression and CUD in mice, since a subgroup of vulnerable and a subgroup of mice resilient to the effects of IRSD can be identified.
- An active coping strategy during episodes of social defeat predicts resilience to the effects of IRSD. Mice with low levels of submissive behavior during episodes of defeat are resilient to the short-and long-term effects of IRSD. They display a greater frequency of grooming and higher social interaction levels than highly submissive mice. In addition, low submissive mice do not acquire CPP.
- Resilience to the short-term effects of IRSD on both social interaction and grooming behavior predicts resilience to the long-term effects of IRSD on cocaine reward.
- The behavioral profile of mice in the EPM is also related with resilience to the effects of IRSD on cocaine reward. Defeated mice that spend a lower percentage of time in the open arms do not acquire CPP.
- The level of novelty-seeking behavior and immobility in the TST do not predict resilience to the effects of IRSD in early adolescence.

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7. ANNEX

Experimental Study 1

Behavioral traits associated with resilience to the effects of repeated social defeat on cocaine-induced conditioned place preference in mice.

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Behavioral Traits Associated With Resilience to the Effects of Repeated Social Defeat on Cocaine-Induced Conditioned Place Preference in Mice

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The relationship between stress and drug use is well demonstrated. Stress-induced by repeated social defeat (RSD) enhances the conditioned place preference (CPP) induced by cocaine in mice. The phenomenon of resilience understood as the ability of subjects to overcome the negative effects of stress is the focus of increasing interest. Our aim is to characterize the behavior of resilient animals with respect to the effects of RSD on the CPP induced by cocaine. To this end, 25 male C57BL/6 mice were exposed to stress by RSD during late adolescence, while other 15 male mice did not undergo stress (controls). On the 2 days following the last defeat, all the animals carried out the elevated plus maze (EPM) and Hole Board, Social Interaction, Tail Suspension and Splash tests. Three weeks later, all the animals performed the CPP paradigm with a low dose of cocaine (1 mg/kg). Exposure to RSD decreased all measurements related to the open arms of the EPM. It also reduced social interaction, immobility in the tail suspension test (TST) and grooming in the splash test. RSD exposure also increased the sensitivity of the mice to the rewarding effects of cocaine, since only defeated animals acquired CPP. Several behavioral traits were related to resilience to the potentiating effect of RSD on cocaine CPP. Mice that showed less submission during defeat episodes, a lower percentage of time in the open arms of the EPM, low novelty-seeking, high social interaction, greater immobility in the TST and a higher frequency of grooming were those that were resilient to the long-term effects of social defeat on cocaine reward since they behaved like controls and did not develop CPP. These results suggest that the behavioral profile of resilient defeated mice is characterized by an active coping response during episodes of defeat, a greater concern for potential dangers, less reactivity in a situation of inevitable moderate stress and fewer depressive-like symptoms after stress. Determining the neurobehavioral substrates of resilience is the first step towards developing behavioral or pharmacological interventions that increase resilience in individuals at a high risk of suffering from stress.

Keywords: resilience, social defeat stress, cocaine, mice, conditioned place preference, reward, vulnerability

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Abbreviations: CPP, conditioned place preference; EPM, elevated plus maze; FG, frequency of grooming; ISI, index of social interaction; NS, novelty-seeking; PND, post-natal day; RSD, repeated social defeat; TI, time of immobility; TST, tail suspension test; %TOA, percentage of time in open arms.

INTRODUCTION

According to the World Health Organization, the global prevalence of cocaine use was estimated at roughly 0.4% of the global population aged 15–64 in 2016 (about 18.2 million users), with higher incidence rates in developed societies (World Drug Report, 2018). Individual and environmental variables act as risk factors, facilitating the initiation and maintenance of drug use, the transition to addiction, and relapse after detoxification (Dellu et al., 1996; Enoch, 2006). Among the environmental factors affecting vulnerability to drug addiction, exposure to stress plays a primary role. Traumatic life events during critical periods of development have a profound influence on the development of personality (Kim et al., 2009; Congdon et al., 2012; Oshri et al., 2013) and increase the risk of suffering from mental and drug-use disorders (Kessler et al., 2010; Sayed et al., 2015).

Chronic social stress, including problems with social interaction (family or friend relationships, work-place stress, bullying, etc.) is the most frequent type of stress faced by human beings. In preclinical studies with rodents, chronic social stress is modeled by the repeated social defeat (RSD) paradigm. Brief episodes of aggression from a more aggressive conspecific, together with social subordination, induce anxiety- and depression-like symptoms (Bartolomucci et al., 2009; Nestler and Hyman, 2010; Hollis and Kabbaj, 2014; Czéh et al., 2016; Vannan et al., 2018). Exposure to RSD has also been shown to increase the rewarding effects of drugs of abuse (Ellenbroek et al., 2005; Burke et al., 2011; Aguilar et al., 2013; García-Pardo et al., 2015, 2017; Newman et al., 2018). Moreover, several studies performed in our laboratory using the conditioned place preference (CPP) paradigm have demonstrated that mice exposed to RSD during late adolescence exhibit an enhanced sensitivity to the rewarding effects of low doses of cocaine in adulthood (Montagud-Romero et al., 2016a,b; Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

In spite of the close relationship between life adversity and psychopathology, not all individuals exposed to stress develop a mental disorder. In fact, most are resilient and display an adaptive response to stress that ensures a relatively normal physical and psychological function (Southwick and Charney, 2012). Thus, resilience can be defined as "the process of adapting well in the face of adversity" (Charney, 2004), or as the capacity to overcome the deleterious consequences of stress, which result in the development of psychiatric disorders in more vulnerable individuals. It is unclear why some individuals are more resilient to the impairing effects of stress than others, but neurochemical, genetic, and epigenetic processes seem to be associated with resilience to stress-related disorders (Cadet, 2016; Osório et al., 2016).

The RSD paradigm has proven to be a useful model for studying the mechanisms involved in susceptibility or resilience to the negative consequences of social stress (Nestler and Hyman, 2010). As in humans, individual differences exist in the development of psychopathology after RSD exposure. Only the subgroup of mice characterized as susceptible to the effects of RSD on social interaction with a conspecific (social avoidance) exhibit a wide variety of deleterious consequences, including anhedonia- and anxiety-like symptoms, elevated reactivity of the hypothalamic-pituitary-adrenal (HPA) axis and other behavioral and physiological alterations (Berton et al., 2006; Krishnan et al., 2007; Nestler and Hyman, 2010; Russo et al., 2012; Russo and Nestler, 2013).

Resilience could also explain why not all individuals who undergo stressful experiences become addicted to drugs of abuse. Using the RSD model, Krishnan et al. (2007) demonstrated that only mice characterized as susceptible (mice that displayed social avoidance after RSD exposure) developed cocaine-induced CPP. Similarly, animals vulnerable to the effects of RSD on social interaction were shown to increase alcohol self-administration in comparison to non-stressed controls or resilient animals that did not develop social avoidance after RSD (Nelson et al., 2018). Both studies suggest that resilient mice that do not display a deficit of social interaction after stress are also resilient to the rewarding effects of drugs of abuse. These are the only studies to have identified animals that were susceptible or resilient to the influence of RSD on the rewarding effects of drugs of abuse. As Cadet (2016) noted, most neuroscience research has focused on identifying negative or pathological elements underlying a subject's vulnerability to drug addiction; however, the characterization of the traits that confer resilience against the consequences of social stress on the effects of drugs of abuse could be a more effective approach to preventing and treating addictive disorders. Identifying predictive behavioral patterns of resilience is the first step towards developing early, individualized preventive strategies that enhance resilience and promote a resilient personality in individuals at risk who are exposed to significative levels of stress.

Thus, the aim of this work was to determine the existence of individual differences in response to RSD and to characterize the behavioral profile of animals that are resilient to the long-term effects of social defeat on cocaine-induced CPP. For this purpose, a group of late adolescent mice were exposed to RSD (four episodes separated by intervals of 72 h), while another group did not undergo stress. The behavior of the defeated mice was evaluated during the first and fourth episodes of defeat and they were segregated in two subgroups according to the time they spent engaged in defense/submission. The short-term effects of RSD were evaluated to compare the behavior of defeated mice to that of control mice in the elevated plus-maze and the hole board and in social interaction, tail suspension and splash tests, 24–48 h after the last episode of defeat. According to the behavior of the defeated mice in these behavioral tests, they were segregated into two subgroups: one affected by RSD (vulnerable mice), and the other behaving like the control group (resilient mice). Three weeks after the last episode of defeat, acquisition of CPP after conditioning with a low dose of cocaine was evaluated in all the mice in order to identify the behavioral traits that confer resilience to the long-term effects of RSD on the CPP induced by cocaine. A lack of CPP was used to define the animals that were resilient to the effects of RSD on cocaine reward since non-stressed mice did not develop CPP with the dose of cocaine employed.

MATERIALS AND METHODS

Subjects

Forty male mice of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were used in the study. They arrived in the laboratory on a postnatal day (PND) 21 and were housed for 26 days before initiation of the experimental procedures. Experimental mice (C57BL/6) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents (OF1) were individually housed in plastic cages (23 × 32 × 20 cm) in order to induce heightened aggression (Rodríguez-Arias et al., 1998). To reduce their stress levels in response to experimental manipulations, grouped mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures. All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available *ad libitum*, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocol was approved by the Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

Drugs

Animals were injected intraperitoneally with 1 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The physiological saline was also used to dissolve the cocaine. The dose of cocaine was selected on the basis of previous studies (Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

Experimental Design

After an adaptation period, the experimental mice (C57BL/6) were assigned to two groups: one non-stressed control group (n = 15) and another subsequently exposed to four episodes of RSD (n = 25) on PND 47, 50, 53 and 56. On PND 57–58, all mice underwent different behavioral tests: elevated plus maze (EPM), hole board, social interaction, tail suspension, and splash tests. Afterward, all mice were housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (see **Figure 1**). All experiments took place during the dark period (8.30–16.30) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing.

Experimental Protocols

Repeated Social Defeat (RSD)

The RSD procedure consisted of four encounters (separated by intervals of 72 h, PND 47, 50, 53 and 56) with a conspecific isolated mouse (OF1), which resulted in the defeat of the experimental animal. Each encounter lasted for 25 min and consisted of three phases, which began by introducing the experimental animal (intruder) into the home cage of the aggressive opponent (resident) for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall, which allowed social interaction and threats from the aggressive male resident. The wire mesh was then removed from the cage and the confrontation between the two mice began and lasted for 5 min. In the third phase, 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. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. The criterion

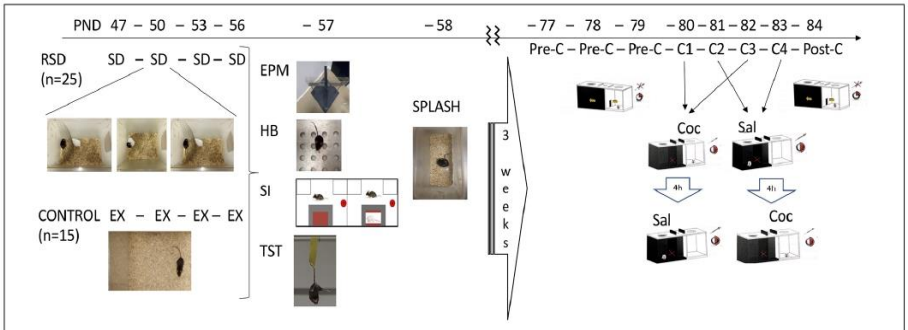

FIGURE 1 | Experimental design. Two groups of mice were used. One group was exposed to repeated social defeat (RSD, n = 25). On postnatal day (PND) 47, 50, 53 and 56, experimental mice were introduced into the cage of an aggressive opponent. The physical contact between them was allowed for only 5 min when the experimental mouse experienced social defeat (SD). On the same PND, the other group (CONTROL, n = 15) explored (EX) an empty cage. On PND 57, all mice performed the elevated plus maze (EPM) and the hole board (HB), social interaction (SI) and tail suspension test (TST). On PND 58, all mice performed the splash test. After an interval of 3 weeks, all mice underwent the conditioned place preference (CPP) paradigm. On PND 77, 78 and 79, they underwent the pre-conditioning (Pre-C) phase. On PND 80, 81, 82, 83 they performed four conditioning sessions (C1-C4) receiving 1 mg/kg of cocaine (Coc) or saline (Sal) before being placed to the drug- or saline-paired compartment, respectively. On PND 84, mice underwent the post-conditioning (Post-C) phase.

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; Ribeiro Do Couto et al., 2006). All experimental mice displayed defeat, given that they all faced resident mice with high levels of aggression. The first and fourth agonistic encounters were videotaped and evaluated by an observer who was blind to the treatment (Brain et al., 1989) using a computerized system (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent in avoidance/flee and defense/submission by the experimental mice and the time spent in threat and attack by the resident aggressive mice were measured, as were the latencies of these behaviors. The control (non-stressed) group underwent the same protocol, without the presence of a "resident" mouse in the cage (exploration).

Elevated Plus Maze (EPM)

The effects of RSD on anxiety were evaluated using the EPM paradigm on PND 57. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments; therefore, it measures the extent to which rodents avoid high open spaces. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and the measurements in the closed arms are lower, and vice versa (Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997). Moreover, the total entries into the closed arms are regarded as locomotor activity scores (Campos et al., 2013; Valzachi et al., 2013).

At the beginning of each trial, subjects were placed on the central platform facing an open arm and were allowed to explore for 5 min. The maze was cleaned with a 7% alcohol swab after each test, and the device remained untouched until completely dry. The behavior of the mice was video recorded and later analyzed by an investigator blind to the experimental conditions, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The measures recorded during the test period were frequency of entries and time spent in each section of the apparatus (open arms, closed arms and central platform). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: the latency to first enter the open arms, the time and percentage of time [(open/open + closed) × 100] spent in the open arms, the number and the percentage of open arm entries and total entries into the arms.

Hole Board Test

The novelty-seeking of mice was evaluated in the hole board test 24 h after the last defeat or exploration (PND 57). This test was carried out in a square box (28 × 28 × 20.5 cm) with transparent Plexiglas walls and 16 equidistant holes of 3 cm in diameter on the floor (CIBERTEC SA, Madrid, Spain). Photocells below the surface of the holes detected the number of times that mice performed a head-dip. At the beginning of the test, mice were placed in the same corner of the box and were allowed to freely explore the apparatus for 10 min. The latency to the first dip and the frequency of dips were automatically recorded by the apparatus.

Social Interaction Test

Twenty-four hours after the last defeat or exploration (PND 57), the social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated plexiglass cage (10 × 6.5 × 30 cm) was placed in the middle of one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions. The first time (object phase), the perforated plexiglass cage was empty. After 10 min of exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was confined to the perforated cage (to safeguard the experimental mouse from attack) and the experimental mouse was reintroduced in the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the RSD episodes). In both phases, the time spent in the 8 cm area surrounding the perforated cage—the interaction zone—was registered and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An index of social interaction (ISI) was obtained [time spent in the interaction zone during the social phase/(time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase); Henriques-Alves and Queiroz, 2016]. The ISI is commonly used as the social preference-avoidance index (Krishnan et al., 2007).

Tail Suspension Test (TST)

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair (Pollak et al., 2010). It is based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung in an uncontrollable fashion by their tail (Cryan et al., 2005). This has been used as a measure of behavioral depression because, when antidepressant treatments are given prior to the test, the subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle (Pollak et al., 2010).

Twenty-four hours after the last defeat or exploration (PND 57), we investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the

TST. Following the protocol described by Vaugeois et al. (1997), mice were suspended by the tail, using adhesive tape, from a hook connected to a strain gauge that recorded their movements during a 6-min test period. The behavior displayed by the mice was video recorded and later analyzed by an observer blind to the treatment received by the animal, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameters considered for the statistical analyses were the total time spent immobile and the latency to show immobility.

Splash Test

The splash test consisted of spraying a 10% sucrose solution on the dorsal coat of a mouse placed in a transparent cage (15 × 30 × 20 cm) with regular bedding to stimulate grooming behavior. The behavior of the mice was videotaped for 5 min and later analyzed by an observer blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The latency to the first grooming, the time spent engaged in this behavior and its frequency were recorded. An increase in the latency of grooming and a decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior (Smolinsky et al., 2009).

Conditioned Place Preference (CPP)

Three weeks after the last episode of social defeat, the animals carried out the CPP procedure. For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 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 recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain).

The CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for detailed explanations of the procedure, see Maldonado et al., 2007). In brief, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15-min period was recorded. Animals showing a strong unconditioned aversion or a preference for a given compartment were excluded from the study. In the second phase (conditioning), which lasted for 4 days, experimental animals received saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, were injected with 1 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 min. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment was recorded during a 15-min period.

Statistical Analysis

The effects of RSD on the different behavioral measures (with the exception of CPP) were evaluated by means of unpaired Student *t*-tests, comparing the non-stressed control group to the defeated group (control vs. RSD). In the case of CPP, a mixed two-way ANOVA with a within-subjects variable Days with two levels (Pre-C and Post-C) and a between-subjects variable Stress with two levels (Control and RSD) was used. *Post hoc* comparisons were performed with Bonferroni tests, which allow multiple hypotheses to be tested simultaneously, limiting the type I error rate without increasing the probability of a type II error occurring.

With the data obtained in the defeat episodes and in the behavioral tests performed 24 or 48 h afterward (EPM, hole board, social interaction, tail suspension and splash tests), the group of defeated mice was separated into two subgroups according to the median of the whole group. Mice with scores higher than the median were assigned to the High Score group and those with lower scores to the Low Score group. For example, defeated mice were defined as high or low novelty-seeking (NS) according to their head-dip scores (below or above the defeated group median) in the hole board test. We have previously used this median-split analysis to study the effects of NS on the behavioral effects of different drugs of abuse (Arenas et al., 2014; Montagud-Romero et al., 2014; Mateos-García et al., 2015; Rodríguez-Arias et al., 2015, 2016). A one-way ANOVA with a between-subjects variable—Group, with three levels (Control, Defeated High Score and Defeated Low Score)—was performed for the following measures: time in defense/submission in the first episode of defeat, percentage of time in the open arms of the EPM, number of dips in the hole board, time of immobility in the TST, and grooming (frequency and time) in the splash test. The *post hoc* comparison was performed with the Tukey test. To determine the possible behavioral markers of resilience to the effects of social defeat on cocaine CPP, a mixed two-way ANOVA with a within-subjects variable—Days, with two levels (Pre-C and Post-C)—and a between-subjects variable—Group, with three levels (Control, Defeated High Score and Defeated Low Score)—was used. *Post hoc* comparisons were performed with Bonferroni tests.

In order to determine whether there was a relationship among the performances of mice in the different procedures, Pearson correlation tests were used. In the case of CPP, the conditioning score (time spent in Post-C minus time spent in Pre-C) was calculated. All statistical analyses were performed with the SPSS program.

RESULTS

Effects of RSD on the CPP Induced by Cocaine

The ANOVA of the CPP data showed a significant effect of the variable Days ($F_{(1,38)} = 5.634$; $p < 0.05$) and the Interaction Days × Stress ($F_{(1,38)} = 4.186$; $p < 0.05$). RSD increased the rewarding effects of cocaine since only defeated mice spent more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). Conversely, mice not exposed to defeat (Control group) did not show CPP (**Supplementary Figure S1**).

Behavioral Profile of Mice During Social Defeats and Resilience to Cocaine CPP

After the behavioral analysis of defeat episodes, defeated mice were divided into two subgroups according to their Defense/Submission scores during the first episode of defeat (below or above the median of the defeated group, 20.11 s, Low or High Defense/S. Student *t*-test showed a significant difference between these two subgroups of defeated mice (Low and High Defense/S) with respect to the Time spent in Defense/Submission in the first episode of defeat ($t_{(26)} = -5.878$; $p < 0.001$).

The behavioral profile of mice during the defeat episodes is related to their subsequent resilience or vulnerability to developing cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the Time spent in submissive behavior during the first episode of defeat showed that the variable Days ($F_{(1,37)} = 11.179$; $p < 0.01$) and the interaction Days × Group ($F_{(2,37)} = 3.297$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons revealed that only the High Defense/S group, which spent more time in defensive/submissive behavior, showed CPP ($p < 0.05$, significantly longer time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the Low Defense/S group (defeated mice that showed less time in defensive/submissive behaviors) did not develop CPP (see **Figure 2**).

Besides the Time spent in Defense/Submission, the behavioral analysis of defeat episodes revealed other differences among the mice that were resilient or vulnerable to the effects of RSD on the CPP induced by cocaine. Student's *t*-tests showed significant differences between the two subgroups of defeated mice in the first episode of defeat with respect to Latency of Submission ($t_{(26)} = 2.322$; $p < 0.05$), Time spent in Flight ($t_{(26)} = 4.519$; $p < 0.001$) and Time receiving Threat from the opponent ($t_{(26)} = -4.01$; $p < 0.001$). Moreover, subgroups of defeated mice showed differences in the fourth episode of defeat in the Time spent in Defense/Submission ($t_{(26)} = -2.075$; $p < 0.05$) and in the Latency of Attack from the opponent ($t_{(26)} = -2.334$; $p < 0.05$). As can be seen in **Figure 3**, the behavioral profile of resilient mice was characterized by lower submission and more avoidance/flee. In addition, they received lower levels of threat but were attacked faster.

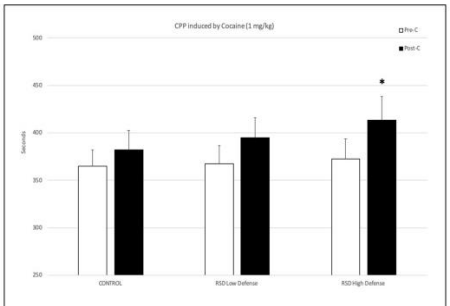

FIGURE 2 | Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice during the first episode of defeat. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The group of defeated mice was divided into two subgroups according to the time spent in defense/submission in the first episode of defeat: RSD Low Defense and RSD High Defense. After defeat, mice were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). *$P < 0.05$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

Elevated Plus Maze and Resilience to Cocaine CPP

RSD induced anxiogenic-like effects in the EPM. Student's *t*-tests showed significant differences between defeated and control mice in several measures related to the open arms. In comparison to controls, mice exposed to RSD showed a decrease in the Time ($t_{(42)} = 3.407$; $p < 0.001$) and Percentage of time ($t_{(42)} = 3.143$; $p < 0.01$) spent in the open arms, an increase in the latency to enter the open arms ($t_{(40)} = -3.174$; $p < 0.01$), and a reduced number of Entries ($t_{(42)} = 5.780$; $p < 0.001$) and Percentage of entries ($t_{(42)} = 3.493$; $p < 0.001$) into the open arms. Furthermore, RSD decreased the total number of Total entries into the arms ($t_{(42)} = 5.410$; $p < 0.001$; **Figure 4**).

In order to evaluate resilience to the effects of RSD in the EPM, defeated mice were divided into two subgroups according to their scores of Percentage of time in the open arms (below or above the median of the defeated group, 25.92%), and Low or High %TOA. A one-way ANOVA showed a significant effect of the variable Group ($F_{(2,41)} = 41.326$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the Low %TOA group was significantly different from the control and High %TOA groups ($ps < 0.001$; **Figure 5A**). Thus, there was a group of mice that were resilient to the effects of RSD on the EPM and did not show a decrease in the percentage of time spent in the open arms.

However, resilience to the anxiogenic-like effects of RSD in the EPM is inversely related to resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of controls and the two groups of defeated mice (Low and High %TOA) showed that the variable Days ($F_{(1,38)} = 8.046$; $p < 0.01$) and the Interaction Days × Group ($F_{(2,38)} = 3.806$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the mice that spent the higher percentage of time in the open arms (High %TOA) developed CPP ($p < 0.001$, more time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the group of defeated mice that spent the lower percentage of time in the open arms (Low %TOA) did not develop CPP (see **Figure 5B**).

Besides the percentage of time in the open arms, there were other differences in the open arm measures between mice that were resilient and vulnerable to the long-term effects of RSD on cocaine-induced CPP. Student's *t*-tests indicated significant differences between both groups of defeated mice with respect to the time spent ($t_{(26)} = -5.937$; $p < 0.001$), number of entries

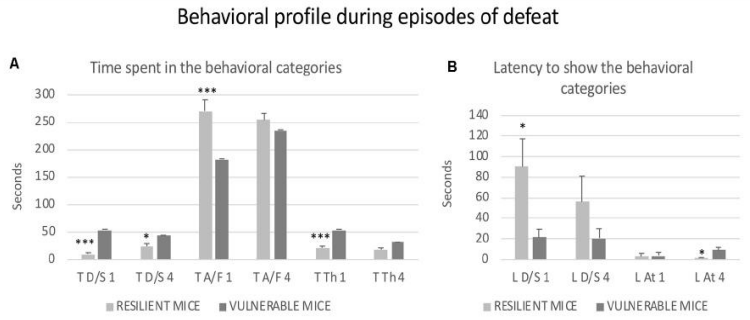

FIGURE 3 | Behavioral profile during episodes of the defeat of mice that were resilient to the long-term effects of RSD on a cocaine-induced CPP. All mice were exposed to RSD (n = 25) and later conditioned with cocaine. **(A)** Bars represent the mean (±SEM) time (in seconds) spent in the behavioral categories of defense/submission (T D/S), avoidance/flee (T A/F) and threat (T Th) analyzed during the first and fourth episodes of defeat. **(B)** Bars represent the mean (±SEM) latency (in seconds) to show defense/submission (L D/S) and attack (L At). As can be seen in **Figure 2**, defeated mice that did not acquire cocaine-induced CPP were defined as resilient mice (light gray bars), while defeated mice that developed cocaine-induced CPP were defined as vulnerable mice (dark gray bars). $^{*}P < 0.05$, $^{***}P < 0.001$, significant difference vs. vulnerable mice.

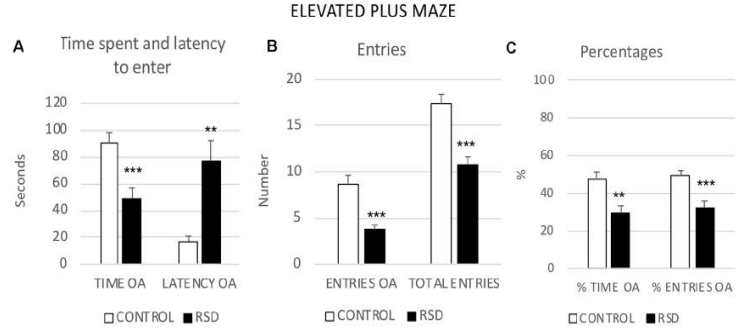

FIGURE 4 | Effects of RSD on the EPM. One group of mice was not exposed to stress (CONTROL, n = 15) and the other group was exposed to RSD (n = 25). The behavior of mice in the EPM was evaluated. **(A)** Bars represent the mean (±SEM) time (in seconds) spent in the open arms (OA) of the maze and the mean (±SEM) latency to enter to the OA. **(B)** Bars represent the mean (±SEM) number of entries into the arms of the maze. **(C)** Bars represent the mean (±SEM) percentages of time spent in and entries into the OA. $^{**}P < 0.01$, $^{***}P < 0.001$, significant difference with respect to CONTROL group.

($t_{(26)} = -3.341$; $p < 0.01$) and percentage of entries into the open arms ($t_{(26)} = -4.619$; $p < 0.001$). It appeared that mice that were resilient to the long-term effects of RSD on cocaine-induced CPP engaged less in the exploration of the open arms (see **Supplementary Figure S2**).

Hole Board Test and Resilience to Cocaine CPP

No significant effects of RSD were observed in the latency to the first dip, but defeated mice showed an almost significant reduction in the number of dips ($t_{(40)} = 1.930$, $p < 0.06$; **Figure 6A**, second bar).

In order to evaluate resilience to the effects of RSD in the hole board test, defeated mice were divided into two subgroups according to their dip scores (below or above the median of the defeated group, 26 dips), Low novelty-seeking (Low NS) or High NS. A one-way ANOVA revealed a significant effect of the variable Group ($F_{(2,39)} = 12.91$, $p < 0.001$). Tukey *post hoc* comcrent from the control group and from the High NS group ($ps < 0.001$; **Figure 6A**). Thus, this group of mice was resilient to the effects of RSD on the hole board test and did not show a decrease in the number of dips.

Resilience to the effects of RSD in the hole board test is inversely related to resilience to the long-term effects of RSD

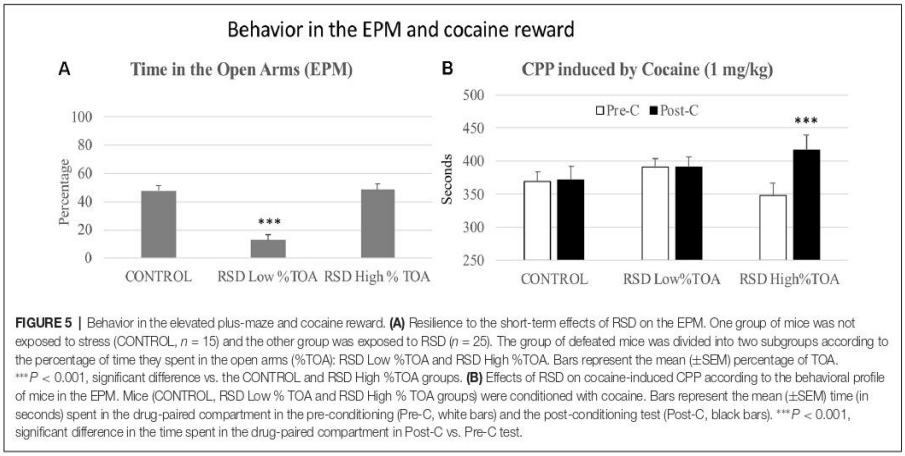

FIGURE 5 | Behavior in the elevated plus-maze and cocaine reward. **(A)** Resilience to the short-term effects of RSD on the EPM. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The group of defeated mice was divided into two subgroups according to the percentage of time they spent in the open arms (%TOA): RSD Low %TOA and RSD High %TOA. Bars represent the mean (±SEM) percentage of TOA. $^{***}P < 0.001$, significant difference vs. the CONTROL and RSD High %TOA groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the EPM. Mice (CONTROL, RSD Low % TOA and RSD High % TOA groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

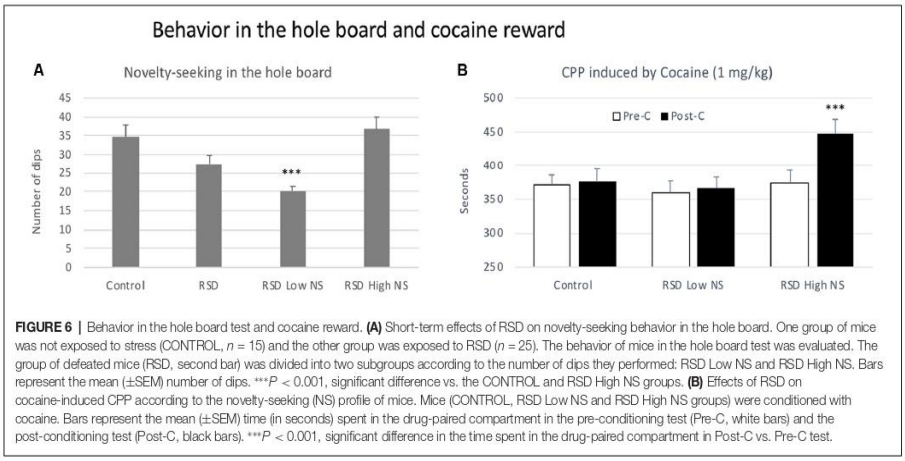

FIGURE 6 | Behavior in the hole board test and cocaine reward. **(A)** Short-term effects of RSD on novelty-seeking behavior in the hole board. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The behavior of mice in the hole board test was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to the number of dips they performed: RSD Low NS and RSD High NS. Bars represent the mean (±SEM) number of dips. $^{***}P < 0.001$, significant difference vs. the CONTROL and RSD High NS groups. **(B)** Effects of RSD on cocaine-induced CPP according to the novelty-seeking (NS) profile of mice. Mice (CONTROL, RSD Low NS and RSD High NS groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the number of dips (Low and High NS) showed that the variable Days ($F_{(1,38)} = 9.41$, $p < 0.004$) and the Interaction Days × Group ($F_{(2,38)} = 3.65$, $p < 0.04$) were significant. Bonferroni *post hoc* comparisons revealed that only mice in the RSD High NS group developed CPP ($p < 0.001$, more time in the drug-paired compartment in Post-C than in Pre-C). The control group (non-defeated mice) and the group of defeated mice with fewer dips (RSD Low NS) did not develop CPP (see **Figure 6B**).

Social Interaction and Resilience to Cocaine CPP

Mice exposed to RSD showed a reduced ISI when they were exposed to an aggressive OF1 mice ($t_{(39)} = 2.924$; $p < 0.01$; **Figure 7A**, second bar). However, this reduction was not observed in all the defeated mice. According to their ISI score (below or above the median of the defeated group, 0.43), defeated mice were separated into two groups: Low ISI or High ISI. A one-way ANOVA revealed a significant effect of the variable Group ($F_{(2,39)} = 42.231$, $p < 0.001$). Tukey *post hoc* comparisons indicated that the Low ISI group was significantly different from

the control and High ISI groups (*p*s < 0.001; **Figure 7A**). Thus, there was a group of defeated mice that was resilient to the impairing effects of RSD on social interaction and that did not engage in less social interaction.

The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in the function of their ISI showed that the variable Days ($F_{(1,37)} = 12.032$; $p < 0.001$) and the interaction Days × Group ($F_{(2,37)} = 3.508$; $p < 0.05$) were significant. *Post hoc* comparisons revealed that only the RSD Low ISI group displayed CPP ($p < 0.05$, significantly higher time spent in the drug-paired compartment in Post-C than in Pre-C). The control group of mice not exposed to defeat and the group of defeated mice that showed a higher social interaction index (RSD High SI group) did not develop CPP (see **Figure 7B**).

Tail Suspension Test and Resilience to Cocaine CPP

With respect to the control group, RSD reduced the Time spent immobile by the mice ($t_{(42)} = 4.452$; $p < 0.0$; **Figure 8A**, second bar), but did not affect the Latency to show this behavior (data not shown).

In order to evaluate resilience to the effects of RSD in the TST, defeated mice were divided into two subgroups according to their scores of Time spent immobile (below or above the median of the defeated group, 141 s, Low TI or High TI. One-way ANOVA showed a significant effect of the variable Group ($F_{(2,41)} = 27.728$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the group that spent less time in immobility (Low TI) was significantly different from the control and High TI groups (*p*s < 0.001;

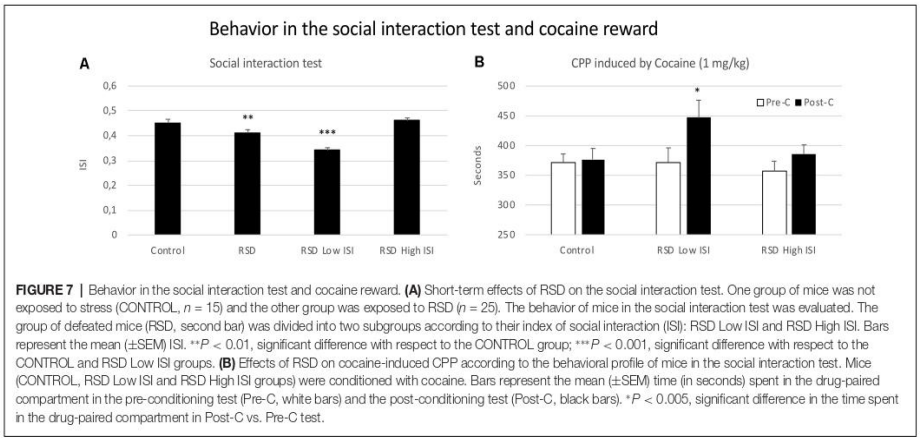

FIGURE 7 | Behavior in the social interaction test and cocaine reward. **(A)** Short-term effects of RSD on the social interaction test. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The behavior of mice in the social interaction test was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to their index of social interaction (ISI): RSD Low ISI and RSD High ISI. Bars represent the mean (±SEM) ISI. $^{**}P < 0.01$, significant difference with respect to the CONTROL group; $^{***}P < 0.001$, significant difference with respect to the CONTROL and RSD Low ISI groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the social interaction test. Mice (CONTROL, RSD Low ISI and RSD High ISI groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{*}P < 0.005$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

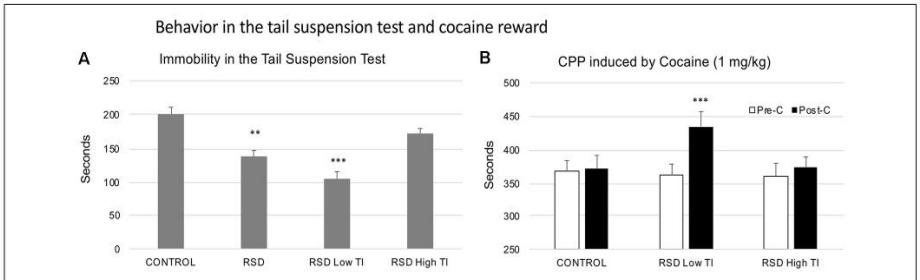

FIGURE 8 | Behavior in the TST and cocaine reward. **(A)** Short-term effects of RSD on the TST. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The behavior of mice in the TST was evaluated. The group of defeated mice (RSD, second bar) was divided into two subgroups according to the time spent immobile (TI): RSD Low TI and RSD High TI. Bars represent the mean (±SEM) time (in seconds) spent immobile. $^{**}P < 0.01$, significant difference with respect to CONTROL group; $^{***}P < 0.001$, significant difference with respect to the CONTROL and RSD High NS groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the TST. Mice (CONTROL, RSD Low TI and RSD High TI groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

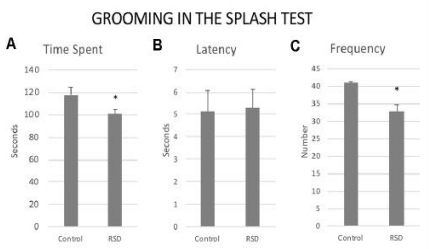

FIGURE 9 | Effects of RSD on grooming behavior in the splash test. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$) on PND 47, 50, 53 and 56. The grooming behavior of mice in the splash test was evaluated. **(A)** Bars represent the mean (±SEM) time (in seconds) spent grooming. **(B)** Bars represent the mean (±SEM) latency (in seconds) to groom. **(C)** Bars represent the mean (±SEM) number of times that the mice perform grooming. $^{*}P < 0.05$, significant difference with respect to Control group.

Figure 8A). Thus, there was a group of mice that was resilient to the effects of RSD on the TST and that did not show a decrease in immobility.

Resilience to the effects of RSD in the tail suspension is associated with resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of the Time spent immobile showed that the variable Days ($F_{(1,38)} = 11.029$; $p < 0.01$) and the Interaction Days × Group ($F_{(2,38)} = 3.320$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the Low TI group developed CPP (more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). The control (non-defeated mice) and the RSD High TI groups did not develop CPP (see **Figure 8B**).

Splash Test and Resilience to Cocaine CPP

Exposure to RSD reduced the Frequency ($t_{(40)} = 2.37$; $p < 0.05$) and the Time spent in Grooming ($t_{(40)} = 2.407$; $p < 0.05$). No significant effects were observed with respect to the Latency to the first grooming ($t_{(40)} = -0.115$; $p < 0.9$; **Figure 9**).

In order to evaluate resilience to the effects of RSD in the splash test, defeated mice were divided into two subgroups according to their scores of Frequency of grooming (below or above the median of the defeated group, 33.8 times), Low FG or High FG. One-way ANOVA showed a significant effect of the variable Group ($F_{(2,39)} = 15, 758$; $p < 0.001$). Tukey *post hoc* comparisons indicated that the group RSD Low FG differed significantly from the control and the RSD High FG groups ($ps < 0.001$; **Figure 10A**). Thus, this group of mice was resilient to the effects of RSD on the splash test and did not show a decrease in grooming.

Resilience to the effects of RSD in the splash test is associated with resilience to the long-term effects of RSD on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of their frequency of grooming showed that the variable Days ($F_{(1,36)} = 7.82$, $p < 0.01$) and the Interaction Days × Group ($F_{(2,36)} = 3.230$; $p < 0.05$) were significant. Bonferroni *post hoc* comparisons showed that only the RSD Low FG group developed CPP (more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$). The control (non-defeated mice) and the RSD High FG groups did not develop CPP (see **Figure 10B**).

When defeated mice were divided into two subgroups according to their scores in Time spent grooming (below or

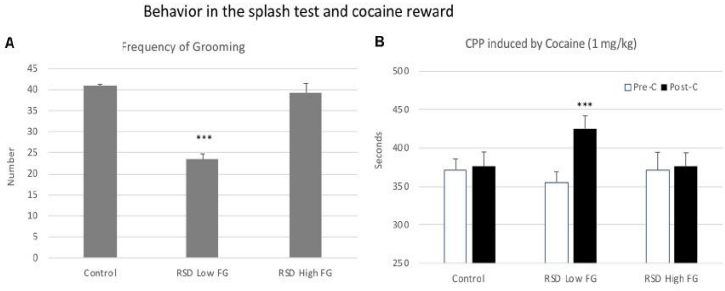

FIGURE 10 | Behavior in the splash test and cocaine reward. **(A)** Resilience to the short-term effects of RSD on grooming behavior in the splash test. One group of mice was not exposed to stress (CONTROL, $n = 15$) and the other group was exposed to RSD ($n = 25$). The grooming behavior of mice in the splash test was evaluated. The group of defeated mice was divided into two subgroups according to their frequency of grooming (FG): RSD Low FG and RSD High FG. Bars represent the mean (±SEM) number of times that the mice performed grooming behavior. $^{***}P < 0.001$, significant difference vs. the CONTROL and RSD High FG groups. **(B)** Effects of RSD on cocaine-induced CPP according to the behavioral profile of mice in the splash test. Mice (CONTROL, RSD Low FG and RSD High FG groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, black bars). $^{***}P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

above the median of the defeated group, 103.22 s), Low TG or High TG, the one-way ANOVA showed a significant effect of the variable Group ($F_{(2,39)} = 16, 32$; $p < 0.001$) and Tukey *post hoc* comparisons indicated that the group RSD Low TG (mean 81.38, SD 3.35) was significantly different from the control (mean 118, SD 6.3) and the RSD High TG (mean 116, SD 4.38) groups ($ps < 0.001$). However, no influence of this behavioral trait on the CPP induced by cocaine was observed. The ANOVA of the CPP data of control, RSD Low TG and RSD High TG showed that only the variable Days was significant ($F_{(1,36)} = 5.53$, $p < 0.05$; data not shown).

Correlations Between Measurements in the Different Behavioral Tests

A limited number of significant correlations were observed among the performances of mice in the different behavioral procedures (see **Supplementary Table S1**). There was a correlation between the percentage of time in the open arms in the EPM and the time spent immobile in the TST ($r = 0.504$; $p < 0.01$), as well as a negative correlation between the time spent in submission and the ISI ($r = -0.403$; $p < 0.05$). The CPP score correlated with the number of dips ($r = 0.421$; $p < 0.05$), with mice with a higher novelty-seeking proving to be more vulnerable to developing cocaine-induced CPP. Furthermore, there was a significant inverse correlation between the ISI and the CPP score ($r = -0.393$, $p < 0.05$), since mice with reduced social interaction were more likely to show CPP.

DISCUSSION

The results of the present work reveal individual differences in the response of mice to RSD exposure during late adolescence. During defeat experiences, some mice displayed less defense/submission and more avoidance/flee behaviors, while others were characterized by the opposite pattern. In the short term, RSD induced anxiety-like symptoms in the EPM, social avoidance in the social interaction test, hyperreactivity in the TST and depressive-like symptoms in the splash test. In the long term, RSD increased the sensitivity of mice to the rewarding effects of a low dose of cocaine in the CPP paradigm. However, only one subgroup of mice showed anxiety- or depression-like symptomatology, a reduction of novelty-seeking, deficits of social interaction, increased reactivity to stress, and greater vulnerability to cocaine-induced CPP (vulnerable mice), while another subgroup remained resilient to the effects of RSD. More importantly, the behavioral profile of the mice in the short-term response to RSD was predictive of subsequent resilience to the long-term influence of RSD on cocaine reward. The defeated mice characterized by lower levels of defensive/submissive behavior, less interest in the open arms in the EPM, less novelty-seeking behavior, a greater level of social interaction, greater immobility in the TST and a higher frequency of grooming in the splash test were resilient to the RSD-induced potentiation of cocaine CPP.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With the Behavioral Profile of Mice During Social Defeat Episodes

After the behavioral analysis of the defeat episodes, defeated mice could be segregated into two subpopulations. In the first episode of defeat, one group (resilient mice) displayed a more active coping response characterized by a longer latency to show submission, less time engaged in defense/submission and more time in avoidance/flee, while the other group (vulnerable mice) showed the opposite behavioral profile. In the fourth episode of defeat, resilient mice showed less defense/submission and were attacked faster by the opponent, which suggests that they managed the stressful situation better than vulnerable mice. The coping response of experimental animals exposed to stress has been used in other studies to distinguish between resilient and vulnerable individuals. For example, male rats were classified as having an active or passive coping strategy according to their latency of submission (Wood et al., 2010, 2013; Pearson-Leary et al., 2017; Grafe et al., 2018; Corbett et al., 2019) and the index of flee behavior in a social interaction test performed after RSD has also been applied to mice (Henriques-Alves and Queiroz, 2016).

Since an active coping strategy has been related with resilience to the negative consequences of stress (Feder et al., 2009; Wu et al., 2013; Wood and Bhatnagar, 2015), we hypothesized that the behavioral profile of mice during defeat episodes is predictive of the long-term effects of RSD on the CPP induced by cocaine. As expected, RSD exposure during late adolescence increased the sensitivity of mice to the rewarding effects of cocaine in adulthood, since only defeated mice developed CPP after conditioning with a dose that was ineffective in non-stressed control mice. These results are in line with and extend our previous findings in OF1 strain mice (Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017; Ferrer-Pérez et al., 2018; García-Pardo et al., 2019). However, this is the first study to demonstrate that certain mice are resilient to the long-term RSD-induced potentiation of cocaine reward. In a previous study, Krishnan et al. (2007) exposed mice to 10 episodes of social defeat and segregated them (on day 11) into susceptible and unsusceptible subjects according to the presence or absence of social avoidance; in other words, only susceptible mice (showing social avoidance) exhibited cocaine-induced CPP. Whether the effect of defeat on cocaine reward continued to be present long after RSD was not evaluated since both resilient and vulnerable mice performed the CPP procedure 24 h after the last session of defeat (Krishnan et al., 2007). Our results indicate that the behavioral profile of late adolescent mice during episodes of defeat is an early predictor of their subsequent susceptibility or resilience to the effects of RSD on cocaine-induced CPP in adulthood. Vulnerable defeated mice with higher levels of submission developed CPP. Conversely, defeated mice that developed a more active coping strategy during defeat episodes were resilient, as they behaved like control mice and did not acquire CPP. These results are in accordance with those observed by Yanovich et al. (2018),

TABLE 1 | Summary table of results.

Behavioral test		Resilient mice	Vulnerable mice
Conditioned place preference	Cocaine CPP	=	↑
Agonistic encounters	Defense/Submission	↓	↑
Elevated plus-maze	Open Arms Measures	↓	=
Hole board test	Novelty-seeking	↓	=
Social interaction test	Social investigation	=	↓
Tail suspension test	Immobility	=	↓
Splash test	Grooming	=	↓

After RSD exposure, vulnerable mice developed CPP with a low dose of cocaine, that did not induce CPP in controls and defeated resilient mice. Reduced defensive/submissive behavior during episodes of defeat, avoidance of the open arms of the elevated plus-maze and lower novelty-seeking were behavioral traits predictive of resilience to the effects of RSD on cocaine CPP. Defeated resilient mice behaved as controls in the social interaction, tail suspension and splash test. Conversely, increased defensive/submissive behavior during episodes of defeat, hyperreactivity in a stressful situation (tail suspension test) and depressive-like behaviors (social avoidance and anhedonia after RSD) were behavioral traits predictive of vulnerability to the effects of RSD on cocaine CPP.

who reported that only selectively bred submissive (but not dominant) mice displayed a marked increase in cocaine CPP after exposure to chronic mild stress. A specific coping strategy is considered to be adaptive (i.e., it reduces the impact of stress on the subject) depending on the environment and the type of stressor (Wood and Bhatnagar, 2015); our results suggest that, in conditions of repeated exposure to brief episodes of social stress, passive coping (such as submissive behaviors and immobility) is less adaptive.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Inversely Related With Resilience to the Anxiety-Like Behavior Induced by RSD in the EPM

Our results show that RSD induces a behavioral profile in the EPM argued to be indicative of anxiety (Campos et al., 2013). In comparison to non-stressed controls, defeated mice spent less time and a lower percentage of time in the open arms of the EPM, performed fewer entries and percentage of entries into these arms, and displayed longer latency to visit an open arm for the first time. These results are in agreement with previous studies reporting that different procedures of social stress induce anxiety-like symptomatology in the EPM (Rodgers and Cole, 1993; Lehmann and Herkenham, 2011; Iñiguez et al., 2014; Duque et al., 2017).

Nevertheless, not all defeated mice showed an aversion for the open arms. Subpopulations could be segregated into those that are susceptible and resilient to the short-term effects of RSD on the EPM. Resilient mice spent a similar percentage of time in the open arms to the control group, which was not exposed to RSD. In contrast, vulnerable mice spent a clearly lower percentage of time in the open arms in comparison to controls and to the other group of defeated mice. Kaufmann and Brennan (2018) also identified a subgroup of defeated mice that spent less time in the open arms (which were also vulnerable to the social avoidance induced by RSD) and another subgroup that was resilient to both deficits. Other studies have also affirmed the existence of animals that are resilient to the effects of several types of social stress on the EPM. For example, using the predator odor stress model, rats were segregated as susceptible or resilient based on EPM behavior and context avoidance (Brodnik et al., 2017). Similarly, in another study, rats were classified as vulnerable or resilient to the effects of RSD on anxiety according to the behavior they displayed in the EPM, dark/lightbox and acoustic startle response test (0 or 1 symptom = resilient rat, 2 or 3 symptoms = vulnerable rat; Le Dorze and Gisquet-Verrier, 2016). In this way, it would seem that some animals are resilient to the anxiety-like behavior induced by social stress.

Due to the close association between anxiety and cocaine use disorders (Vorspan et al., 2015), it can be hypothesized that subjects that are resilient to the effects of RSD on anxiety in the EPM are also resilient to the long-term effects on cocaine reward. However, our results do not support this theory. Unexpectedly, the defeated mice that did not develop cocaine CPP were those that spent a lower percentage of time in the open arms. In contrast, the defeated mice spending a higher percentage of time in the open arms (which were, thus, resilient to the short-term effects of social defeat) showed an enhanced vulnerability to cocaine and developed CPP. No previous studies have evaluated whether the behavioral profile in the EPM after exposure to RSD is related to subsequent vulnerability or resilience to developing cocaine-induced CPP. Krishnan et al. (2007) did not observe a relationship between the expression of anxiety-like symptoms in the EPM and the acquisition of cocaine-induced CPP in mice exposed to RSD. In the study in question, vulnerable mice (which displayed social avoidance and cocaine-induced CPP) and resilient mice (that did not show these effects) exhibited an increase in the time spent in the closed arms in the EPM (Krishnan et al., 2007). Conversely, in a more recent study, rats that were vulnerable to the stress induced by exposure to the odor of a predator were more sensitive to the effects of cocaine (Brodnik et al., 2017). Seven days after stress exposure, male rats were segregated into resilient or susceptible groups according to the time they spent in the open arms of the EPM and in the compartment associated with the predator's odor. In comparison to resilient rats, the hyperactivity induced by cocaine and the reinforcing effect of this drug in the self-administration paradigm were enhanced in susceptible rats (Brodnik et al., 2017). These divergent results may be due to differences in the methodology (species, type of stress, the time elapsed between stress exposure and behavioral testing, etc.). However, from our point of view, the most

important factor is the criterion used to discriminate resilient animals from vulnerable animals. In the study by Brodnik et al. (2017), rats were considered vulnerable when they met both criteria: less than 50 s in the open arms and less than 20 s in the odor-associated compartment. In this way, it can be assumed that rats showing an anxiety/fear response to stress are more vulnerable to the effects of cocaine. Conversely, in the present study, mice that spent a lower percentage of time in the open arms of the EPM were resilient to the long-term effects of RSD and did not develop cocaine-induced CPP. It is not logical to assume that the mice with higher anxiety levels were less vulnerable to cocaine; thus, we propose other interpretations of the results obtained. The EPM test not only reveals an anxious state but might also suggest behavioral disinhibition. In this sense, the longer time spent in the open arms by vulnerable mice that developed CPP might indicate a pre-existing impulsive phenotype (Gass et al., 2014) that predisposes them to be more vulnerable to the effects of cocaine. Furthermore, it is important to consider that the EPM entails a conflict between two natural tendencies: the motivation to stay in the protected closed arms, naturally associated with safety, and the motivation to explore the non-protected open arms, which may be associated with a potential danger or a threat (Ennaceur and Chazot, 2016). There is no objective evidence as to the real significance of a reduction in the open arms measures: i.e., whether it represents anxiety or a sense of security. From our point of view, the mice that were resilient to the long-term effects of RSD on cocaine reward were those that, after experiencing an attack from an opponent, actively avoided the open arms to stay safe from other potential threats.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Related With the Novelty-Seeking Profile of Defeated Mice in the Hole Board Test

Exposure to RSD induced a reduction in the number of dips in the hole board test in some defeated mice, since a tendency to such reduction was observed only in the group of defeated mice as a whole ($n = 25$, $p = 0.06$ with respect to controls). In rodents, novelty-seeking behavior has been defined as a "preference for" or a tendency to increase the exploration of novel objects and environments (Nadal-Alemany, 2008; Belin et al., 2011; Vidal-Infer et al., 2012). A very limited number of studies have evaluated the influence of stress exposure on novelty-seeking behavior, and the few data reported are controversial. In male rats, RSD did not modify their behavior in the hole board test 24 h after the last defeat (Albonetti and Farabollini, 1994), but chronic RSD reduced directed exploration in mice (Erhardt et al., 2009). Conversely, rats chronically exposed to predator odor before and during puberty showed increased novelty-seeking during late adolescence (Toledo-Rodriguez and Sandi, 2011). Such discrepant results are probably due to the different developmental periods in which the animals were exposed to stress. From our point of view, the lower number of dips in the hole board test in the subgroup of defeated mice could have been due to the fact that RSD induced an emotional arousing state that motivated a reduced exploration of a novel, potentially dangerous environment.

The influence that the novelty-seeking trait exerts on vulnerability to stress and drug use has been repeatedly demonstrated (Kabbaj et al., 2001; Duclot et al., 2011; Vidal-Infer et al., 2012; Duclot and Kabbaj, 2013; Clinton et al., 2014; Hodges et al., 2018). In particular, novelty-seeking behavior is one of the personality factors that may explain individual differences in vulnerability to drug abuse (Dellu et al., 1996). Higher novelty-seeking has been identified as a risk factor for the initiation of drug use and transition to abuse (Kelley et al., 2004; Staiger et al., 2007; Milivojevic et al., 2012; Mateos-García et al., 2015). In line with this idea, we observed that the subgroup of mice showing greater novelty-seeking after RSD was more vulnerable to the rewarding effects of cocaine. Conversely, mice performing a significantly lower number of dips (that is, mice that responded to RSD with emotionality or avoidance of a novel environment) remained resilient to the long-term effects of RSD on cocaine reward and did not develop CPP. These results, together with those observed in the EPM, lead us to assume that defeated mice that avoid potential risk are protected from the subsequent consequences of social stress on the rewarding effects of cocaine.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With Resilience to the Social Avoidance Induced by RSD in the Social Interaction Test

Exposure to RSD produced a short-term deficit of social interaction. This reduction of the ISI in defeated mice has been associated with the social avoidance that characterizes affective disorders (Golden et al., 2011), and has been repeatedly observed after RSD or social instability (Krishnan et al., 2007; Golden et al., 2011; Henriques-Alves and Queiroz, 2016; Browne et al., 2018; Dong et al., 2018; Hodges et al., 2018). Furthermore, the ISI is the most used measure to distinguish between mice that are resilient or vulnerable to the effects of different models of social defeat (Krishnan et al., 2007; Chaudhury et al., 2013; Donahue et al., 2014; Friedman et al., 2014; Hodes et al., 2014; Isingrini et al., 2016; Sun et al., 2016; Nelson et al., 2018; Prabhu et al., 2018; Gururajan et al., 2019). In this line, we have also observed a subgroup of resilient mice (with similar ISI to that of control mice) and another subgroup of vulnerable mice that displayed social avoidance. It must be taken into account that the type of opponent used in the social interaction test has an influence on the results observed. In the present study, the use of the OF1 strain instead of the strain employed as experimental animals probably induced a more pronounced social avoidance in defeated mice. In fact, it has been reported that, when the target in the social interaction test was a C57BL/6J mouse, both susceptible and resilient mice spent more time in the interaction zone than when the opponent was an aggressive CD1 mouse (Han et al., 2014). Notwithstanding, even when the opponent was of the same strain, the social interaction was significantly higher in resilient than in susceptible mice (Han et al., 2014).

Defeated mice resilient to social avoidance were also resilient to the long-term effects of RSD on cocaine reward. Only the subgroup of defeated mice with a deficit of social interaction developed CPP after conditioning with a low dose of cocaine that was ineffective in non-stressed control mice and in resilient defeated mice. Similar results have been observed by Krishnan et al. (2007), who reported that only mice with a deficit of social interaction (susceptible) developed CPP after conditioning with 5 mg/kg of cocaine 24 h after social defeat, while unsusceptible mice without social avoidance did not develop CPP. Similarly, vulnerable mice with lower levels of social interaction showed reduced alcohol self-administration in comparison to control mice not exposed to stress and to resilient animals without a social interaction deficit (Nelson et al., 2018).

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With the Resilience to Hyperreactivity Induced by RSD in the Tail Suspension Test

Exposure to RSD reduced the amount of time spent immobile in the TST, an unexpected result taking into account that immobility in this test has been considered to be depression-like behavior (Katz, 1982; Cryan et al., 2005; Pollak et al., 2010). However, other studies have shown that stressed mice spent less time being immobile than control mice in the tail suspension (Brockhurst et al., 2015) or in the forced swim tests (Suo et al., 2013; Sadler and Bailey, 2016). In contrast, other researchers have reported that RSD did not affect immobility 24 h after the last episode of defeat (Kinsey et al., 2007; Krishnan et al., 2007), or even increased it in defeated mice identified as vulnerable in a social interaction test (Dong et al., 2017). As Commons et al. (2017) stated, the behavioral alterations observed in the TST must be interpreted with caution, since this paradigm may model the stress-coping strategy from which depressive-like behavior is inferred. Besides, the use of the TST can be problematic in the case of C57BL/6 mice, as they have a propensity to climb using their tails (Can et al., 2012). In the present study, the decrease in immobility in defeated mice could be attributed to inoculation against stress; however, we suspect that such an effect is related to an enhanced reactivity of defeated mice to the situation of moderate inescapable stress that the TST represents. In contraposition to the conventional interpretation of immobility in the forced swim and TSTs as behavioral despair (Katz, 1982), it has been understood by some to represent enhanced anxiety (van Dijken et al., 1992). In support of this idea, a subgroup of defeated mice exhibiting less immobility in the TST reduced their consumption of sucrose, a behavior associated with the lack of interest in pleasurable activities that characterizes depression (Bowens et al., 2012). In the same line, we observed that RSD decreased the frequency of grooming in the splash test, an effect interpreted as depressive-like symptomatology (see the following section). Considered together, these results suggest that the decreased immobility of defeated mice in the TST should be interpreted as an enhanced reactivity to this stressful situation, rather than a reduction of depressive-like behavior.

In addition, our results indicate that vulnerable mice that are more immobile in the TST are more sensitive to the rewarding effects of cocaine and CPP acquired with a low dose of this drug. Conversely, resilient mice with immobility values similar to controls and not exposed to stress did not develop CPP. Thus, mice that were resilient to RSD-induced hyperreactivity were also resilient to the long-term effects of RSD on cocaine reward.

Resilience to the Long-Term Effects of RSD on Cocaine CPP Is Associated With Resilience to Depressive-Like Behavior Induced by RSD in the Splash Test

Exposure to RSD decreased the duration and frequency of grooming in the splash test, considered a relevant measure of the motivational state of animals (Butelman et al., 2019). A reduction of grooming behavior has been observed after exposure to different stressors (Jolles et al., 1979; Spruijt et al., 1992; Charney, 2004; Smolinsky et al., 2009; Heaney et al., 2011; Veloso et al., 2016; Szewczyk et al., 2019), and has been interpreted as anhedonia, as it is reversed by antidepressant drugs (Brachman et al., 2016; de Souza et al., 2019).

In addition, we have observed that some defeated mice remained resilient to the depressive-like behavior induced by RSD. Although there are no studies with the splash test, Krishnan et al. (2007) demonstrated that mice that were resilient to the effects of RSD in the social interaction test were also resilient to depression-like behavior evaluated with the sucrose preference test. Conversely, a recent study that segregated mice into resilient and vulnerable subjects according to their immobility values in the TST showed that vulnerable mice with higher immobility spent more time engaged in grooming and exhibited this behavior more frequently in an unfamiliar cage (Reis-Silva et al., 2019). A possible explanation for these divergent results is the different type of stressor used (RSD vs. tail suspension) and the controversial interpretation of the results obtained in the TST (as commented before, greater immobility has been interpreted as depression-like behavior and as lower reactivity to moderate inescapable stress). In the present study, the resilience to the short-term effects of RSD on the frequency of grooming predicted subsequent resilience to cocaine reward; only vulnerable mice with reduced grooming behavior acquired cocaine-induced CPP 3 weeks after RSD. Similar results were reported by Krishnan et al. (2007) 1 day after the last episode of defeat, as only mice with anhedonia (indicated by a lower sucrose preference) showed cocaine CPP.

Correlation Between Behavioral Markers of Resilience to the Long-Term Effects of RSD on the CPP Induced by Cocaine

As discussed in previous sections, the segregation of experimental animals into vulnerable or resilient subpopulations with respect to the effects of stress on cocaine reward has been the subject of only two studies. Brodnik et al. (2017) observed that the reinforcing efficacy of cocaine in the self-administration paradigm was lower in mice that were resilient to the effects of stress (predator odor exposure) on EPM behavior and context

avoidance. Previously, Krishnan et al. (2007) had reported that mice resilient to the effects of RSD on social interaction were also resilient to developing anhedonia and cocaine-induced CPP a short time after defeat. They also observed that the resilient phenotype regarding social interaction (but not regarding depressive-like behavior) persisted 4 weeks after defeat; however, the potential long-term enhanced vulnerability to the rewarding effects of cocaine was not evaluated (Krishnan et al., 2007). The results of the present work are in accordance with and extend those obtained in the aforementioned studies. Our main contribution is to demonstrate that some behavioral profiles of the short-term response to social stress predict the subsequent resilience of defeated mice to the rewarding effects of cocaine. Resilient mice that did not develop cocaine CPP were less submissive during defeat episodes, a behavioral profile associated with an active coping with stress (Finnell et al., 2017; Pearson-Leary et al., 2017; Grafe et al., 2018), which in turn is associated with resilience to developing mental disorders. Furthermore, resilient mice avoided the open arms of the EPM and showed less novelty-seeking in the hole board test, which can be interpreted as active avoidance-risk behavior (in concordance with the higher avoidance/flee behavior observed during the first defeat episode). Mice resilient to developing cocaine CPP were also resilient to social avoidance in the social interaction test, hyperreactivity in the TST and depressive-like behavior in the splash test.

We have attempted to establish a potential association of the different resilient phenotypes by means of correlations between the variables shown to be indicative of resilience to the long-term effects of RSD on cocaine reward (lower defense/submission, lower percentage of time in open arms, lower novelty-seeking, higher ISI, higher immobility in the TST and higher frequency of grooming). Furthermore, the contribution of each individual variable to cocaine resilience was determined by correlating these variables with the CPP score. There was a correlation between the time spent in submission and the ISI: the mice that showed less submission during the defeat episodes were resilient to developing a deficit of social interaction. The percentage of time in the open arms of the EPM correlated with the time spent immobile in the TST; thus, the behavior in both tests seemed to be associated in some way. In light of these results, we hypothesize that mice that are less reactive to stress (i.e., those that show more immobility) feel less of a need for safety in the EPM. The number of dips negatively correlated (although non-significantly, $p < 0.073$) with the frequency of grooming, which may indicate that mice that respond to social defeat with lower novelty-seeking are also more resilient to developing anhedonia. With respect to the CPP scores, only two correlations were statistically significant. First, the correlation between CPP score and the number of dips indicated that the novelty-seeking profile was a strong predictor of resilience or vulnerability to the rewarding effects of cocaine. Second, the correlation between CPP score and ISI indicated that social avoidance induced by RSD was associated with enhanced vulnerability to the rewarding effects of cocaine. These correlations suggest that resilience to the effects of social defeat on cocaine reward may be a result of particular behavioral traits or the combination of several behavioral traits. An important fact is that most of the behavioral tests used in the present study measure unrelated behaviors. However, even in the absence of a correlation with the CPP score, the response of defeated mice in each one of these behavioral tests was predictive of its subsequent resilience or vulnerability to cocaine reward. The main relevance of these results is that they show that cocaine use disorders should be considered from a multi-dimensional perspective. Such disorders result from the interaction of biological and behavioral processes that are altered by environmental factors, such as stress exposure. Some individual behavioral traits, such as the level of novelty-seeking or the degree of social interaction, may confer, by themselves, an enhanced or reduced responsivity to cocaine reward. However, more frequently, a complex neurobehavioral profile resulting from the combination of two or more behavioral traits contributes in a cumulative way to resilience or vulnerability to developing a drug addiction.

CONCLUSION

In the present study, we demonstrate that resilience to the long-term potentiation of the rewarding effects of cocaine-induced RSD is associated with different behavioral profiles. Resilient mice are characterized by less submission during defeat episodes, less interest in the open arms in the EPM, lower novelty-seeking, less reactivity in the TST, and an absence of RSD-induced deficits such as social avoidance and anhedonia (see **Table 1**). A limitation of the present work is the use of the median to discriminate between vulnerable and resilient mice in the behavioral procedures. With this approach, we defined as resilient any mouse below or above the median depending on the test and variable used. However, it is certainly improbable that 50% of the subjects were constantly resilient to the different effects of social defeat stress. In future studies, we will employ larger samples of defeated mice and quartiles (rather than the median) to divide them into resilient and non-resilient subjects, in order to give a more substantiality to the notion of resilience.

The general conclusion of this study, based on the data from all the tests performed, is that several individual traits, including an active coping response, and avoidance of potential dangers in unknown environments, and reduced acute stress reactivity, contribute to a subject's resilience to the negative consequences of social stress (deficit of social interaction, anhedonia and enhanced drug sensitivity). From a translational point of view, our results support the real-world observation that not all individuals exposed to social stress during late adolescence subsequently suffer from mental disorders. For example, not all adolescents exposed to bullying develop cocaine use disorders in adulthood. Resilient subjects have less probability of showing symptoms of post-traumatic stress disorder after a traumatic event (Tugade and Fredrickson, 2004; Wrenn et al., 2011; Lee et al., 2014), while more vulnerable subjects can suffer from mental disorders and addictive behaviors in response to this level of stress. In this context, it is necessary to promote in vulnerable individuals attitudes and personality traits that are characteristic of resilience. According to our results, and to evidence in humans, an active coping strategy (Feder et al.,

2009) and a search for social support (Wu et al., 2013) should be encouraged. Individuals with an active coping response attempt to change their perception of the stressful stimulus (Wu et al., 2013) by means of cognitive reevaluation, which may increase positive thinking, another individual factor associated with resilience (Meredith et al., 2011; Holz et al., 2019). In addition, it is necessary to decrease reactivity to stressful events and increase awareness of dangers, as well as to promote the self-control function and sense of safety. These can be achieved by means of problem-solving tasks, relaxation training and cognitive restructuration (Thompson et al., 2018).

Future works should address ways to increase resilience in vulnerable animals. The negative consequences of stress can be reduced through environmental manipulations (Greenwood and Fleshner, 2008; Schloesser et al., 2010; MacKay et al., 2017) and by allowing mastication during stress exposure, a model of active behavioral coping in rodents (Hennessy and Foy, 1987; Hori et al., 2004; Kubo et al., 2009; Stalnaker et al., 2009; Helmreich et al., 2012). Finally, it is important to study the neurobiological substrates of resilience, which underly the behavioral phenotypes observed in our study. There are recent reviews about the causes of resilience that highlight the importance of neuroplasticity in several brain networks, changes at the blood-brain barrier, genetic factors, and the role of the immune system, the metabolism and the gut microbiota (Cathomas et al., 2019; Feder et al., 2019; Holz et al., 2019; Tsyglakova et al., 2019; Turkson et al., 2019). Based on previous studies in our laboratory, we propose that a reduced inflammatory response, epigenetic changes (lower histone acetylation activity), reduced permeability of the BBB, and lower glutamate activity in the brain reward system may mediate the phenotype of resilience to the effects of RSD on cocaine reward (Montagud-Romero et al., 2016a, 2017; Rodríguez-Arias et al., 2017; García-Pardo et al., 2019). Understanding the individual traits and the neurobiological mechanisms that promote resilience may give rise to multiple new approaches to prevention and the development of pharmacological or behavioral interventions that can increase resilience to the negative sequelae of stress and their influence on drug addiction and other mental disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

AUTHOR CONTRIBUTIONS

MA and MG-P contributed to the conception and design of the study. MA, CC-L, MM-C, and AS-O performed the experiments, organized the databases and performed the statistical analyses. CC-L, MM-C, and AS-O wrote sections of the manuscript. MG-P wrote the complete first draft of the manuscript. MA wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Experimental Study 2

Intermittent voluntary wheel running promotes resilience to the negative consequences of repeated social defeat in mice.

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Intermittent voluntary wheel running promotes resilience to the negative consequences of repeated social defeat in mice

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ABSTRACT

A novel approach to reduce the incidence of substance use disorders is to promote resilience to stress using environmental resources such as physical exercise. In the present study we test the hypothesis that Voluntary Wheel Running (VWR) during adolescence blocks the negative consequences of stress induced by intermittent repeated social defeat (IRSD). Four groups of adolescent male C57BL/6 mice were employed in the experiment; two groups were exposed to VWR (1 h, 3 days/week) from postnatal day (PND) 21 until the first social defeat (PND 47), while the remaining two groups did not have access to activity wheels (controls). On PND 47, 50, 53 and 56 mice, who had performed VWR, were exposed to an episode of social defeat by a resident aggressive mouse (VWR+IRSD group) or allowed to explore an empty cage (VWR+EXPL group). The same procedure was performed with control mice that had not undergone VWR (CONTROL+IRSD and CONTROL+EXPL groups). On PND 57, all the mice performed the Elevated Plus Maze (EPM), Hole-Board, Social Interaction, Tail Suspension and Splash tests. After an interval of 3 weeks, all mice underwent a conditioned place preference (CPP) procedure with 1 mg/kg of cocaine. Exposure to VWR prevented the negative consequences of social stress in the EPM, splash test and CPP, since the VWR+IRSD group did not display anxiety- or depression-like effects or the potentiation of cocaine reward observed in the Control+IRSD group. Our results support the idea that physical exercise promotes resilience to stress and represents an excellent target in drug abuse prevention.

1. Introduction

Stress is part of our lives and necessary for survival, but chronic stress can induce negative consequences. In this context, it is important to note that each person copes with stress differently and that responses to adversity vary among individuals. While some people develop psychiatric conditions after stressful experiences, such as major depressive disorder or post-traumatic stress disorder, others recover from stress without presenting significant symptoms [1]. The phenomenon of resilience, understood as the ability of subjects to overcome the negative effects of stress, is the focus of the present work.

It is well-known that social stress plays an important role in the incidence of several mental diseases, including the development of addictive behaviors. Numerous reports have demonstrated that exposure to intermittent repeated social defeat (IRSD), an animal model of social stress, results in a significant increase in the rewarding effects of different drugs of abuse, such as MDMA [2], alcohol [3] or cocaine [4–6]. Cocaine use disorder is a pressing problem with limited therapeutic options, and it is of enormous importance to understand the impact of social stress on the development of this disorder and find ways to promote resilience to the negative effects of stress. In a recent study in our laboratory, we observed that mice exposed to IRSD performed differently in a series of behavioral tests compared to mice that had not suffered stress. In particular, exposure to IRSD increased anxiety-like behavior in the elevated plus maze (EPM) and reactivity in the tail suspension test (TST), as well as reducing social interaction and grooming behavior in the splash test - indicative of depressive-like behavior - and increasing the rewarding effects of cocaine in the conditioned place preference paradigm (CPP) [7]. However, in the same study, we also demonstrated that some defeated mice did not go on to develop cocaine CPP. Several behavioral traits were associated with resilience to the effects of IRSD on cocaine reward. First, resilient defeated mice exhibited a behavioral profile characterized by an active coping response (less submission) during episodes of defeat. Additionally, in the short-term period following IRSD, resilient mice showed greater aversion to potential dangers (lower novelty-seeking in the

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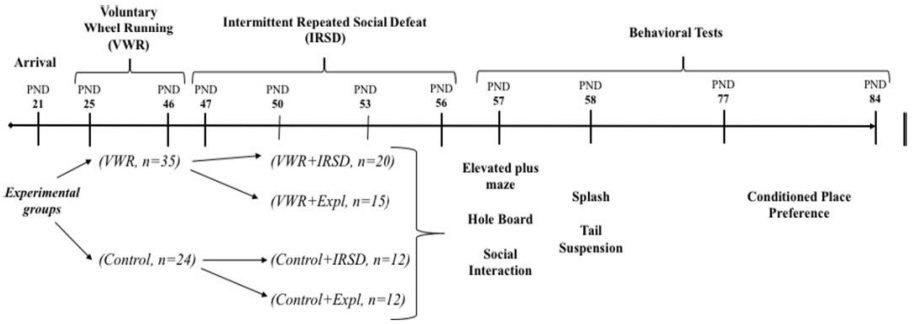

Fig. 1. Timeline of experimental protocols and experimental design.

hole-board test), as well as displaying less reactivity in a situation of inevitable moderate stress (e.g. the TST) and fewer depressive-like symptoms after stress (less social avoidance in the social interaction test and a lack of anhedonia in the splash test) [7]. Surprisingly, this subgroup of defeated mice which did not develop CPP was also characterized by a lower percentage of time spent in the open arms of the EPM than both the non-stressed mice and another subgroup of defeated mice that were resilient to the IRSD-induced anxiety [7].

A growing amount of research is currently focused on the development of pharmacological and environmental strategies that promote resilience in order to prevent the development of stress-related mental disorders, including cocaine addiction. Among possible environmental manipulations, physical exercise has been demonstrated to prevent and improve the pathophysiology of illnesses and to promote healthy aging [8]. Voluntary Wheel Running (VWR) is a rodent model that mimics aspects of human physical exercise and can be effective in reducing cocaine CPP [9] and promoting resilience to the effects of chronic SD stress in mice [10,11]. In particular, sedentary mice exposed to stress were shown to develop a depressive-like state, characterized by anhedonia and social avoidance, whereas stressed mice that had engaged in wheel running showed resilience to these effects of stress [10]. Similarly, VWR was found to reverse the impairment of social preference and the deficiency of social interaction induced by exposure to chronic social defeat [11]. In this line, Reguilon and colleagues showed that VWR reverses the increase in ethanol intake induced by IRSD [12].

Thus, the aim of the present study was to test the hypothesis that physical activity promotes the development of resilience to the negative effects of IRSD; namely, the development of anxiety and depression-like behavior and a potentiation of the rewarding effects of cocaine. We endorse VWR as an effective tool to prevent the short- and long- term detrimental effects of social stress.

2. Materials and methods

2.1. Animals

A total of 59 male of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were delivered to our laboratory on postnatal day (PND) 21. All mice (except those used as aggressive opponents) were housed in groups (4–5 mice per cage) in plastic cages (25 × 25 × 14.5 cm). All the mice housed in the same cage underwent the same experimental conditions and the composition of each cage remained stable throughout the whole study. Mice employed as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression [13]. To reduce the animals' stress levels in response to experimental manipulations, grouped mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures.

All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available ad libitum, except during behavioral tests. Mice were fed a standard chow diet (Teklad Global Diet 2014, ENVIGO rms, Spain) containing 14% protein and 4% fat. All mice were provided by shredded paper strips and wooden gnawing sticks in their home cage in order to increase their welfare. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocol was approved by the Ethics Committee of Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1549371980205, 2019-VSC-PEA-056).

2.2. Drugs

Animals were injected intraperitoneally with 1 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The same physiological saline was also used to dissolve the cocaine. The dose of cocaine administered was selected based on previous studies [5,7,14], which have shown that it induces CPP in mice exposed to IRSD but not in naive mice.

2.3. Experimental design

Four groups of mice were used for this study. On PND 25–46, a set of mice ($n = 35$) performed a physical activity protocol (VWR) over a total of 11 h (1 h, 3 days per week: Monday, Wednesday and Friday) while another set of mice (Control) did not engage in physical activity ($n = 24$). On PND 47, 24 h after the last session of VWR, the mice that had engaged in physical activity were assigned to one of two groups: one group was subsequently exposed to four episodes of social defeat (IRSD on PND 47, 50, 53 and 56) in the cage of a resident mice (VWR+IRSD, $n = 20$), while the other group did not undergo stress and explored an empty cage on the same days (VWR+EXPL, $n = 15$). Control mice, which did not undergo the VWR protocol, were also assigned to two groups: one group was exposed to IRSD on PND 47, 50, 53 and 56 (Control+IRSD, $n = 12$), and the other group explored an empty cage on the same days (Control+EXPL, $n = 12$). A lower number of mice were used in these groups because we have previously evaluated and confirmed the effects of IRSD and EXPL (7).

On PND 57–58, all mice underwent a series of behavioral tests: elevated plus maze (EPM), hole-board (HB), social interaction (SI), splash (SH) and tail suspension (TS) tests. On PND 57, the mice

performed first the EPM, followed by the HB and then the SI test, with an interval of 1 h between each test. On PND 58, mice performed the SH test and, after an interval of 1 h, the TS test. The order of tests was based on a previous study carried out in our laboratory (7), according to the degree of stress that the tests had induced in the mice; in this way, we hoped to prevent previous experience in a test from affecting the performance in subsequent tests. As the open arms measurements are very sensitive to environmental conditions and prior manipulation of the animal, we decided to perform the EPM first. And, as the TS is the most stressful test, it was performed last. Afterwards, all mice were housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (PND 77–84) in order to evaluate the long-term effects of the experimental manipulations undergone during adolescence on cocaine reward in the adult mice (see Fig. 1). All experiments took place during the dark period (8.30 h–16.30 h) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing. During the behavioral tests the experimental room was illuminated with a dim red light (40 lux at 1 m above floor level).

2.4. Experimental protocols

2.4.1. Voluntary wheel running (VWR)

Eight low-profile running wheels (Med Associates Inc.) were employed in the experiments. Each wheel, made entirely of plastic (10.25 × 15.5 × 13.7 cm), rotates on a central axis in a horizontal plane, allowing physical activity through spontaneous locomotion. All animals in the running condition (VWR+IRSD and VWR+EXPL groups) were distributed in batches of eight and allowed to run individually on the wheel (which was located in a plastic cage different to the home cage) for 1 h, three times per week (Monday, Wednesday and Friday). Control animals (Control+IRSD and Control+EXPL groups) were placed in the same plastic cages (different from their own) without any running wheel. A total of 11 VWR sessions took place (see Fig. 1). The procedure and duration of VWR was based on the work of Reguilon and colleagues, who also studied the effects of IRSD on vulnerability to drugs of abuse, in particular to alcohol [12]. The cohort of 35 VWR mice were exposed to the wheels during 5 rounds between 9.30 h and 15.00 h. The mice included in each round varied every day according to a standardized schedule (for example, mice in the first round on the first day were exposed to the wheels in the second round on the second day, and so on, successively).

2.4.2. Intermittent repeated social defeat (IRSD)

The IRSD procedure consisted of four encounters, separated by intervals of 72 h (PND 47, 50, 53 and 56), between an experimental C57BL/6 mouse (intruder) and an isolated OF1 mouse (resident), which culminated in the defeat of the experimental animal (IRSD groups). Each encounter lasted for 25 min and consisted of three phases, which began by introducing the experimental animal into the home cage of the aggressive opponent and leaving it there for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall, which allowed social interaction and threats from the aggressive resident male. The wire mesh was then removed from the cage and confrontation between the two mice initiated and was allowed to last for 5 min. In the third phase, 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. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. 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 [15–17]. All the experimental mice displayed defeat and submission postures, given that they all faced resident mice with high levels of aggression. Previous studies in our laboratory have demonstrated that this protocol of IRSD is effective in increasing corticosterone levels and inducing behavioral alterations related with stress [2,7,18]. The non-stressed mice underwent the same protocol but without the presence of a "resident'' mouse in the cage; in other words, they simply explored the cage (EXPL groups) (see Fig. 1).

2.4.3. Elevated plus maze (EPM)

The effects of IRSD on anxiety were evaluated on PND 57 using the EPM paradigm. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments; in this way, it measures the extent to which rodents avoid high open spaces. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of white Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and those in the closed arms are lower, and vice versa [19,20]. The total number of entries into the arms is a score of locomotor activity scores [21,22].

At the beginning of each trial, subjects were placed on the central platform facing an open arm and allowed to explore for 5 min. The maze was cleaned with a 7% alcohol swab after each test and left to dry completely. The behavior of the mice was video recorded and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: the latency to first enter the open arms; the time and percentage of time [(open / open + closed) × 100] spent in the open arms; and the number and percentage of open arm entries and total number of entries into the arms.

2.4.4. Hole board test

The mice's novelty-seeking was evaluated in the hole board test 24 h after the last defeat or exploration (PND 57). This test was carried out in a square box (28 × 28 × 20.5 cm) with transparent Plexiglas walls and 16 equidistant holes with a diameter of 3 cm in the floor (CIBERTECSA, Madrid, Spain). Photocells below the surface of the holes detected the number of times a mouse performed a head-dip. At the beginning of the test, mice were placed in the same corner of the box and were allowed to freely explore the apparatus for 10 min. The latency to the first dip and the frequency of dips were automatically recorded by the apparatus.

2.4.5. Social interaction test

Twenty-four hours after the last defeat or exploration (PND 57), the social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated plexiglass cage (10 × 6.5 × 30 cm) was placed in the middle of one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions.

The first time (object phase), the perforated plexiglass cage was empty. After 10 min of exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was placed inside the perforated cage (which safeguarded the experimental mouse from attack) and the experimental mouse was reintroduced into the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the IRSD episodes). In both phases, the time spent in an 8 cm wide corridor surrounding the perforated cage—the interaction zone—was automatically registered using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). As in Henriques-Alves and Queiroz

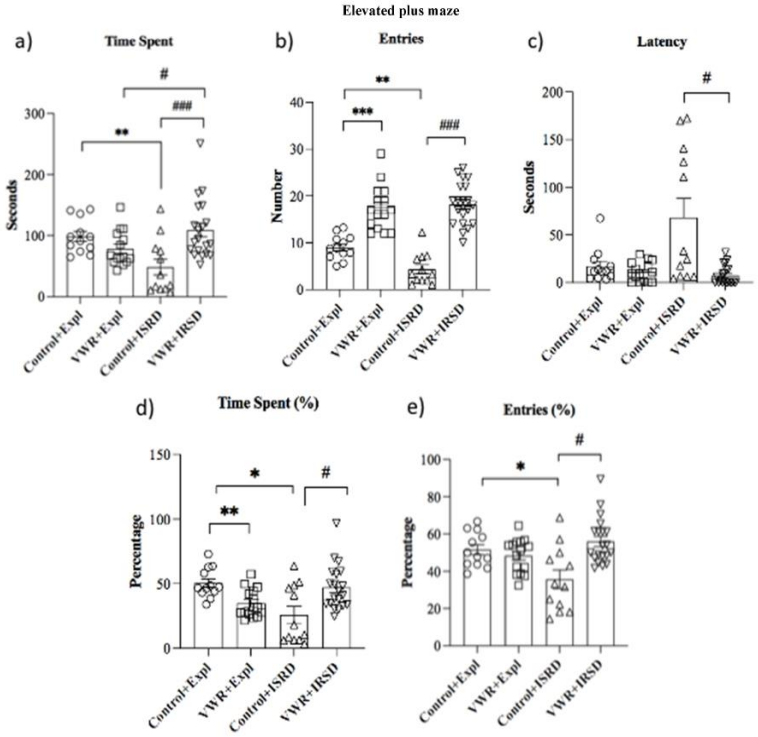

Fig. 2. Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) in the elevated plus maze. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) during late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were subjected to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. (a) Time spent in the Open Arms (OA). Bars represent the mean (±SD) time spent by each group in the OA of the maze. $^{**}p < 0.01$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, $\#\#\#p < 0.001$, significant difference with respect to the VWR+IRSD group; (b) Number of entries into the OA. Bars represent the mean (±SD) number of entries into the OA for each group. $^{**}p < 0.01$, $^{***}p < 0.001$, significant difference with respect to the Control+Expl group; $\#\#\#p < 0.001$, significant difference with respect to the VWR+IRSD group; (c) Latency to enter the OA. Bars represent the mean (±SD) latency to enter the OA for each group. $\#p < 0.05$, significant difference with respect to the VWR+ISRD group; (d) Percentage of time spent in the OA. Bars represent the mean (±SD) percentage of time spent by each group in the OA.$^{*}p < 0.05$, $^{**}p < 0.01$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, significant difference with respect to the VWR+IRSD group; (d) Percentage of entries into the OA. Bars represent the mean (±SD) percentage of entries into the OA for each group. $^{*}p < 0.05$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, significant difference with respect to the VWR+ISRD group.

[23], an index of social interaction (ISI) was obtained [time spent in the interaction zone during the social phase/ (time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase). The ISI is commonly used as the social preference-avoidance index [24].

2.4.6. Splash test

The splash test consisted of spraying a 10% sucrose solution (to stimulate grooming behavior) on the dorsal coat of a mouse that had been placed in a transparent cage (15 × 30 × 20 cm) containing bedding. The behavior of the mice was videotaped for 5 min and later analyzed by an observer who was blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent engaged in grooming and the frequency of this behavior were recorded forty-eight hours after the last defeat or exploration (PND 58). A decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior [25].

2.4.7. Tail Suspension test (TST)

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair [26]. It is based on the observation that rodents, after initial escape-oriented movements, adopt an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung by their tail so that they are immobile [27]. This is used as a measure of behavioral depression because, when antidepressant treatments are administered prior to the test, subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle [26].

Forty-eight hours after the last defeat or exploration (PND 58), we

investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the TST. Following the protocol described by Vaugeois and colleagues, mice were suspended by the tail (using adhesive tape) from a hook during a 6 min test period [28]. The behavior displayed by the mice was video recorded and later analyzed by an observer who was blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameter considered for the statistical analyses was the total time spent immobile.

2.4.8. Conditioned Place preference (CPP) paradigm

Three weeks after the last episode of social defeat, the animals underwent the CPP procedure. For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 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 recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain).

CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for detailed explanations of the procedure, see [29]). In brief, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15 min period was recorded on 3 consecutive days. Animals showing a strong unconditioned aversion (less than 250 s) or a preference (more than 650 s) for a particular compartment in the last pre-conditioning session were excluded from the study ($n = 3$). In the second phase (conditioning), which lasted 4 days, experimental animals received saline before being confined to the vehicle-paired compartment for 30 min. Subsequently, after an interval of 4 h, they were injected with 1 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 min. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment was recorded during a 15 min period.

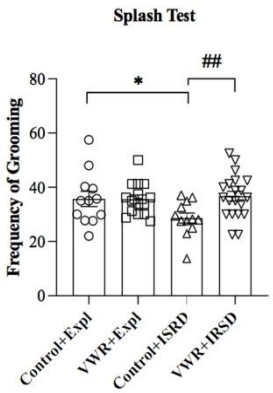

Fig. 3. Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) on the splash test. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 58. Bars represent the mean (±SD) frequency of grooming in each group. *$p < 0.05$, significant difference with respect to the Control+Expl group; ##$p < 0.01$, significant difference with respect to the VWR+IRSD group.

2.5. Statistical analysis

The data of the behavioral tests were first analyzed by means of a Levene test to check the variance of data. Levene's tests confirmed homogeneous variance among the data for: time spent in [$F(3,55) = 1.913$; $p > 0.05$] and entries into [$F(3,55) = 1.730$; $p > 0.05$] the open arms of the EPM; number of dips in the hole board test [$F(3,55) = 1.533$; $p > 0.05$]; frequency of grooming in the splash test [$F(3,55) = 0.895$; $p > 0.05$]; and length of time spent immobile in the TST [$F(3,55) = 0.294$; $p > 0.05$]. Conversely, they revealed unequal variance among the data for: percentage of time spent in [$F(3,55) = 5.260$; $p < 0.05$] and percentage of entries into [$F(3,55) = 2.915$; $p < 0.05$] the open arms of EPM; total entries into the arms [$F(3,55) = 5.506$; $p < 0.05$] of the EPM; and ISI in the social interaction test [$F(3,55) = 3.293$; $p < 0.05$]. The behavioral effects of VWR and IRSD on time spent in/entries into the open arms of the EPM, number of dips in the hole board test, frequency of grooming in the splash test and length of time immobile in the TST (data sets with homogeneous variance) were evaluated using a two-way ANOVA with two between-subjects variables; Voluntary Wheel Running, with two levels (Control and VWR), and Defeat, with two levels (Expl and IRSD). Post hoc comparisons were performed with Bonferroni tests, which allow multiple hypotheses to be tested simultaneously, thus limiting the type I error rate without increasing the probability of a type II error occurring. The behavioral effects of VWR and IRSD on percentage of time spent in and percentage of entries into the open arms of EPM, total entries into the arms of the EPM, and ISI in the social interaction test (data sets with unequal variance) were analyzed by means of an ANOVA with one variable - Group (with four levels: Control+Expl, VWR+Expl, Control+IRSD and VWR+IRSD) - and post-hoc comparisons with the Games-Howell Test. The effects of VWR and IRSD on the CPP paradigm (data set with homogeneous variance) were evaluated using a three-way ANOVA with the between-subjects variables Voluntary Wheel Running and Defeat (described above) and a within-subjects variable; Days, with two levels (Pre-C and Post-C). Post hoc comparisons were performed with Bonferroni tests. All statistical analyses were carried out with the SPSS program.

3. Results

3.1. Physical activity prevented the anxiogenic effects of IRSD in the EPM

The ANOVA of the time spent in the open arms of the EPM revealed that the Interaction of the variables Stress X Running was significant [$F(1,55) = 14.405$; $p < 0.001$] (Fig. 2a). Post-hoc comparisons showed that control mice exposed to defeat (Control+IRSD) spent less time in the open arms of the EPM than mice in the Control+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.001$) groups. In addition, mice in the VWR+IRSD group spent more time in the open arms than those in the VWR+Expl group ($p < 0.05$).

The ANOVA of the number of entries into the open arms of the EPM revealed that the variable Running [$F(1,55) = 104.223$; $p < 0.001$] and the Interaction of the variables Stress X Running [$F(1,55) = 6.182$; $p < 0.05$] were significant (Fig. 2b). Mice engaging in physical activity performed more entries into the open arms of the EPM than control mice ($p < 0.001$). Post-hoc comparisons of the Interaction showed that control mice exposed to defeat (Control+IRSD) performed less entries into the open arms than those in the Control+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.001$) groups. In addition, mice in the VWR+Expl group performed more entries into the open arms than mice in the Control+Expl group ($p < 0.001$).

The ANOVA of the latency to enter the open arms of the EPM was

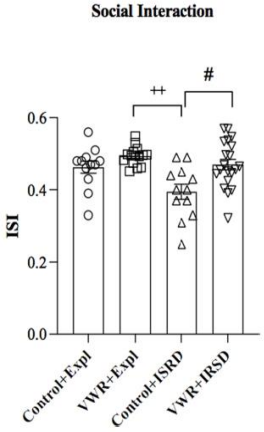

Fig. 4. Effects of Voluntary Wheel Running (VWR) and Intermittent Repeated Social Defeat (IRSD) on the Social Interaction Test. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice engaging in Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. Bars represent the mean (±SD) index of social interaction (ISI) in each group. ++ $p < 0.01$, significant difference with respect to the VWR+EXPL group; #$p < 0.05$, significant difference with respect to the VWR+IRSD group.

significant [$F(3,55) = 9.796$; $p < 0.001$] (Fig. 2c). Post-hoc comparison showed that the VWR+IRSD group displayed a lower latency to enter the open arms than the Control+IRSD group ($p < 0.05$).

The ANOVA of data of the percentage of time spent in the open arms of the EPM was significant [$F(3,55) = 6.509$; $p < 0.001$] (Fig. 2d). Post-hoc comparison showed that the groups VWR+Expl and Control+IRSD spent a lower percentage of time in the open arms of the EPM than mice in the Control+Expl ($p < 0.01$ and $p < 0.05$, respectively); in addition, the Control+IRSD group spent a lower percentage of time in the open arms than mice in the VWR+IRSD group ($p < 0.05$).

The ANOVA of the percentage of entries into the open arms of the EPM was significant [$F(3,55) = 7.013$; $p < 0.001$] (Fig. 2e). Post-hoc comparison highlighted a lower percentage of entries into the open arms by mice in the Control+IRSD group than by mice in the Control+Expl ($p < 0.05$) or VWR+IRSD ($p < 0.05$) groups.

The ANOVA of the total entries into the arms of the EPM was significant [$F(3,55) = 64.988$; $p < 0.001$] (data not shown). Post-hoc comparison showed that the Control+IRSD group performed a lower total number of entries into the arms than the rest of the groups (ps < 0.001); in addition, the VWR+IRSD and VWR+Expl groups performed a higher number of total entries into the arms than the Control+Expl group (ps < 0.001).

3.2. Physical activity prevented the depression-like effects of IRSD in the splash test

The ANOVA of the frequency of grooming revealed that the Interaction of the variables Stress X Running was significant [$F(1,55) = 4$; $p < 0.05$] (Fig. 3). Post-hoc comparison showed a lower frequency of grooming in the Control+IRSD group than in the Control+Expl ($p < 0.05$) and VWR+IRSD ($p < 0.01$) groups.

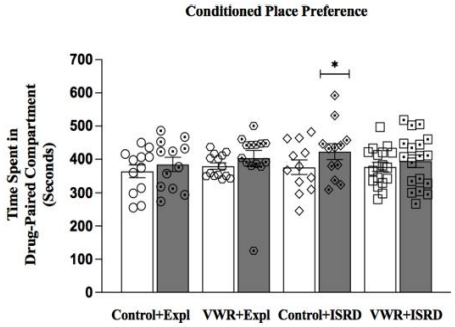

Fig. 5. Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) on the conditioned place preference (CPP) paradigm. Naïve control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). After behavioral tests on PND 57–58 and an interval of 3 weeks, mice were conditioned with cocaine (1 mg/kg). Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, black bars) and post-conditioning (Post-C, gray bars) tests. *$p < 0.05$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.3. Physical activity prevented the social interaction deficit induced by IRSD

The ANOVA of the ISI data was significant [$F(3,55) = 7.057$; $p < 0.001$] (Fig. 4). Post-hoc comparison showed that the group Control+IRSD had a lower ISI than the VWR+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.05$) groups.

3.4. Physical activity did not modify the effects of IRSD in the hole-board and tail suspension test

The ANOVA of the number of dips revealed Stress to be the only significant variable [$F(1,55) = 5.211$; $p < 0.05$] (data not shown). Mice exposed to defeat showed a lower number of head dips than mice that were not exposed to stress ($p < 0.05$).

The ANOVA of the time spent in immobile in the tail suspension test revealed that only the variable Stress was significant [$F(1,55) = 10.045$; $p < 0.01$] (data not shown). Mice exposed to defeat spent less time immobile than those not exposed to stress ($p < 0.01$).

3.5. Physical activity prevented the potentiation of cocaine CPP induced by IRSD

The ANOVA of the time spent in the drug-paired compartment revealed that only the variable Days was significant [$F(1,52) = 6.519$; $p < 0.05$] (Fig. 5). Although the Interaction Days X Stress X Running was not significant, post-hoc comparisons showed that mice in the Control+IRSD group spent more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.05$). Additional Student t tests comparing the time spent by each group in the drug-paired compartment in Pre-C vs Post-C confirmed a significant difference only in the Control+IRSD group ($p < 0.05$).

4. Discussion

The present study reveals that physical activity during adolescence prevents some of the short-term effects of subsequent exposure to social stress in late adolescent mice, such as the induction of anxiogenic- and depressive-like effects in the EPM and the splash test, respectively, and the development of social avoidance in the social interaction test. In addition, we have seen how VWR during adolescence also prevents the long-term effects of IRSD on drug reward; in particular, the potentiation of cocaine-induced CPP in adult mice. Thus, our results indicate that physical activity during adolescence can enhance resilience to the short- and long-term negative consequences of subsequent social stress.

Influence of VWR on the short-term behavioral effects of IRSD

Exposure to IRSD increased anxiety in the EPM, which is in line with previous studies carried out in our laboratory [7,30]. Similarly, other research using the chronic SD stress (CSDS) paradigm has demonstrated that male mice exposed to defeat on 5–10 consecutive days display anxiety-like symptoms [31–39]. Regarding the influence of VWR on the anxiogenic effects of IRSD, we observed that physical activity during adolescence prevented the reduction in open arm measurements induced by social stress. Interestingly, this effect of VWR on defeated mice was observed despite the fact that mice exposed only to VWR (VWR+EXPL group) spent a higher percentage of time in the open arms and more time in the closed arms with respect to the Control+Expl group. These results suggest that physical activity, while inoculating against the anxiogenic effects of a subsequent stressor, can itself slightly increase anxiety. Although we have not found any study in the literature concerning changes in corticosterone after limited VWR, there are reports that continuous exposure to VWR for 4–5 weeks increased corticosterone levels in comparison to control mice that did not run and induced changes in the hypothalamic-pituitary-adrenal system [40,41]. The fact that VWR also increased the number of entries into the open arms appears to be related with an increase of general activity, since both the groups engaging in VWR performed a higher number of total entries. Only one previous study has evaluated the influence of physical activity on the anxiety-like effects of social defeat [42]. As in the present work, the authors reported that Syrian hamsters engaging in physical activity 21 days before defeat (three 5 min defeat sessions) exhibited less risk assessment behavior in the EPM than defeated hamsters (controls, without running), which was indicative of lower anxiety. However, no significant differences were observed between the two groups in the time or percentage of time spent in the open arms of the EPM. In addition, socially defeated hamsters exposed to physical activity showed less defensive/submissive behaviors than control/defeated hamsters when confronted by a resident aggressor [42]. In the same line, another study demonstrated that engaging in treadmill activity for two weeks after defeat (30 min daily for 7 days) reversed anxiety-like behavior (in the EPM, light-dark and open field tests) induced by social defeat in rats [43]. All these results, together with those of the present study, suggest that VWR promotes resilience to the anxiogenic effects of social defeat stress.

The social interaction deficit is probably the most studied behavioral consequence of CSDS exposure and has been used to model the social avoidance that characterizes depression in human beings [38,44,45]. In accordance with this, we observed that exposure to IRSD induced such a deficit, similarly to previous studies carried out in our laboratory [7,46]. We also found that exposure to VWR was effective in preventing social avoidance induced by IRSD. Although no previous studies have evaluated the effects of physical activity on the social interaction deficit induced by IRSD, our results are in line with those observed in mice exposed to other procedures of defeat, in which VWR prevented the social avoidance induced by 5 [47] or 10 consecutive days of defeat (CSDS) [10,11,35,48].

In the present research, exposure to IRSD also induced depression-like symptoms in the splash test; in particular, an increase in the latency to perform grooming behavior, in line with previous results obtained by our group showing that defeated mice exhibit a decrease in the frequency of grooming [7] or an increase in the latency to perform this behavior [46]. We also observed that exposure to physical activity prevented the effects of IRSD in the splash test. Similar results were reported by Mul and colleagues, who found that mice engaging in VWR for 21 days before CSDS did not develop the reduction in sucrose preference observed in defeated mice [10]. Similarly, using a 7-day protocol of vicarious SD, Kochi and colleagues reported that rats with trauma witness spent more time immobile in the forced swim test than non-defeated rats, an effect indicative of depression that was absent in rats given access to the VWR for 14 days before vicarious SD [49]. Conversely, we have not observed a preventive effect of VWR on the changes induced by defeat in the tail suspension test. However, it must be taken into account that, in our model, exposure to IRSD did not increase immobility in said test, as would have been expected if such an effect were indicative of depression-like behavior. In contrast, as in a previous study carried out in our laboratory, IRSD decreased the time spent immobile in the tail suspension test, an effect that we interpret as representing a higher level of reactivity of defeated mice to a subsequent stressful situation [7]. In the same way, VWR did not prevent the effects of IRSD on the hole-board test, since a reduction of novelty-seeking was observed in both groups of defeated mice, irrespective of whether or not they had engaged in physical activity. These results are in accordance with those of a recent study in our laboratory, which demonstrated that exposure to a brief period of maternal separation – which has been shown to prevent other effects of IRSD - did not modify the reduction in novelty-seeking induced by exposure to defeat [46].

Influence of VWR on the long-term effects of IRSD on cocaine reward

As expected, adult mice exposed to IRSD during late adolescence displayed enhanced sensitivity to the rewarding effects of cocaine in the CPP paradigm, since they spent more time in the compartment paired with a low dose of cocaine (1 mg/kg) - which was ineffective in inducing CPP in non-stressed mice - in the Post-C vs. Pre-C test. This result confirms our previous observations that exposure to IRSD potentiates the rewarding effects of different drugs of abuse, including cocaine [5,7,14,18,46,50], MDMA [2] and alcohol [51]. Similar results have been observed with animals exposed to the IRSD or CSDS paradigms, in which defeated rats and mice have been shown to develop enhanced sensitivity to different drugs of abuse [52,53].

The VWR procedure employed in our study induced resilience to the potentiating effects of IRSD on cocaine reward, since mice that performed voluntary physical activity before exposure to IRSD did not develop CPP in adulthood. No previous studies have evaluated the influence of VWR during adolescence on the long-term effects of social stress. However, in line with the present results, it has been reported that mice exposed to VWR during and after IRSD are protected against the increase in ethanol consumption induced by IRSD [12]. In addition, in the absence of stress, 1 or 4 weeks of VWR after cocaine conditioning was shown to abolish CPP in mice [9,54], while 6 weeks of VWR lowered breaking points in the cocaine self-administration paradigm [55], and post-extinction VWR attenuated priming-induced reinstatement of cocaine self-administration [56]. However, in other studies, 3 weeks of VWR before cocaine conditioning did not modify CPP in mice [57], while VWR did not prevent stress-induced reinstatement of cocaine seeking [56]. Considered as a whole, these results suggest that running acts as an alternative reinforcer to cocaine; in fact, it has been well demonstrated that physical activity is a motivating and rewarding behavior for rodents [58,59]. A potential enriching component of VWR has also been hypothesized in order to explain the inhibition of cocaine CPP in mice exposed to physical activity [9]. As in the study of Lespine and Tirelli [57], mice in the present study performed VWR during adolescence and underwent cocaine CPP in adulthood, but there is an important difference between the two studies; namely, the exposure to social stress to which our animals were submitted. Our results indicate that, though it did not modify the rewarding effects of cocaine, physical activity exerted a preventive effect on the negative long-term

consequences of social stress for cocaine reward.

One limitation of our study is the use of a single dose of cocaine. However, it must be taken into account that one of the aims was to test whether exposure to VWR can prevent the potentiation of cocaine reward induced by IRSD. In order to observe this potentiation, we needed to use a subthreshold dose of cocaine that was ineffective in inducing CPP in non-stressed mice. If we had used a moderate-to-high dose, it is probable that all the groups would have acquired cocaine CPP due to the strong rewarding effects of this drug. In such a case, we would probably have observed differences between groups regarding the maintenance or reinstatement of CPP. In this regard, we have previously seen that exposure to IRSD prolonged drug-induced CPP and enhanced the vulnerability of mice to priming-induced reinstatement [2,60,61]. We would also expect VWR to prevent these effects of IRSD. This hypothesis should be tested in future studies.

Potential mechanisms underlying the protective effects of VWR on social stress

In the present study we have shown that VWR during adolescence prevents anxiety- and depression-like behaviors (including social avoidance), as well as undermining the potentiation of cocaine reward induced by exposure to IRSD. Regarding the mechanisms underlying the protective influence of VWR on the negative consequences of defeat stress, we hypothesize the involvement of different neurotransmitter systems, neuroplasticity, and neuroinflammatory and epigenetic processes. Physical activity induces changes in different markers of neurotransmitter systems in reward-related brain areas that are also affected by stress and drugs of abuse, such as the dopaminergic and glutamatergic systems [62–64]. For example, VWR reverses the decrease in levels of tyrosine hydroxylase in the ventral tegmental area and of DA D2 receptors in the nucleus accumbens shell induced by CSDS [11]. VWR increases the production of new neurons [54,65], activates BDNF/TRKB signaling in the hippocampus [36,66,67], and induces ΔFosB and altered dendritic morphology in the nucleus accumbens [10], and reverses the neuroinflammatory response induced by IRSD [12]. In addition, exposure to physical activity after CSDS counteracts the increase in oxidative stress through epigenetic mechanisms, including acetylation of histone H3 and modulation of methyl-CpG-binding in the hippocampus [43]. In the same line, when lactate (a metabolite produced during physical activity) is administered before each episode of CSDS, it has been shown to promote resilience to stress by restoring the activity of hippocampal H1 deacetylase (HDAC2/3) [68]. It is important to take into account that most of these studies have employed continuous access to a running wheel, while the limited and intermittent VWR applied in the present study can induce different neurobiological adaptations that underly its protective influence on the negative consequences of defeat stress.

5. Conclusions

CSDS (defeat on several consecutive days) is the most frequently used animal model to study the negative consequences of social stress, such as anxiety- and depression-like symptoms [38,44] and enhanced sensitivity to drugs of abuse [53]. In the same way that most humans exposed to stress do not develop mental disorders, chronically defeated rodents respond to CSDS differently; some develop anxiety-, depression- and addiction-like symptoms, whereas others remain resilient to stress [38,50]. In our laboratory, we have used a different protocol of defeat in mice consisting of intermittent exposure to an episode of defeat that is repeated four times, every 72 h. With this protocol, we have seen how mice exposed to IRSD exhibit an increase in anxiety- and depression-like behavior, an alteration of social interaction, and an impairment of learning [7,30], as well as enhanced sensitivity to drugs of abuse [2,7,14,51]. In addition, we have observed that some mice are vulnerable, while others are resilient to the effects of IRSD stress [7], and that a brief stressful event during childhood (i.e., an acute 6 h episode of maternal separation) can induce resilience to some effects of subsequent IRSD exposure, including depression-like behavior in late adolescence and potentiation of cocaine reward in adulthood [46]. In the present study we have demonstrated that exposure to VWR, a preclinical model of human voluntary exercise [58], also enhances the resilience of mice and protects them against the anxiogenic effects, social interaction deficit, depression-like behavior and potentiation of the rewarding effects of cocaine observed in mice under social stress. A comparison of the behavioral profile of the VWR+IRSD mice in the present study and that of the resilient mice in our previous study is provided in Table 1. Our results endorse the idea that physical activity can prevent the development of stress-related disorders, both in animals and in humans. Nevertheless, it is necessary to carry out more research to determine other protective factors that promote the development of resilience to stress during childhood or early adolescence.

Table 1
Behavioral profile of VWR+IRSD and resilient mice. = mice showed similar values to mice without exposure to social defeat. ↓ mice showed lower values than mice without exposure to social defeat.

	VWR+IRSD mice	Resilient defeated mice
Time in open arms (EPM)	=	↓
Dips (Hole-board)	↓	↓
ISI (Social interaction test)	=	=
Grooming (Splash test)	=	=
Immobility (TST)	↓	=
Cocaine reward (CPP)	=	=

In summary, our findings suggest that physical activity during adolescence is an excellent tool to improve resilience to the negative effects of subsequent social stress on an individual's vulnerability to mental and addictive disorders later in life. Future studies should be conducted to disentangle the mechanisms underlying VWR-promoted stress prevention. In addition, it is important to include females in studies of resilience to social stress, as their response to VWR or other potentially preventive environmental manipulations may differ from that observed in males. Such research could contribute to developing new approaches aimed at avoiding mental stress-related disorders and new therapeutic strategies for treating people at risk of developing a drug use disorder following stressful experiences.

Author contributions

MAA and MPG-P contributed conception and design of the study; CC-L and MAM-C performed the experiments, organized the databases and performed the statistical analyses; CC-L and MAM-C wrote some sections of the manuscript, CC-L and MPG-P wrote the complete first draft of the manuscript and MAA wrote the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Experimental Study 3

Brief Maternal Separation Inoculates Against the Effects of Social Stress on Depression-Like Behavior and Cocaine Reward in Mice.

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Brief Maternal Separation Inoculates Against the Effects of Social Stress on Depression-Like Behavior and Cocaine Reward in Mice

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Exposure to intermittent repeated social defeat (IRSD) increases the vulnerability of mice to the rewarding effects of cocaine in the conditioned place preference (CPP) paradigm. According to the "inoculation of stress" hypothesis, a brief period of maternal separation (MS) can provide protection against the negative effects of IRSD. The aim of the present study was to assess whether exposure to a brief episode of MS prevents the subsequent short-term effects of IRSD on depression- and anxiety-like behaviors and to explore its long-term effects on cocaine CPP in mice. Four groups of male C57BL/6 mice were employed; two groups were separated from their mother [6 h on postnatal day (PND) 9], while the other two groups were not (controls). On PND 47, 50, 53 and 56, mice that had experienced MS were exposed to social defeat in the cage of an aggressive resident mouse (MS + IRSD group) or were allowed to explore an empty cage (MS + EXPL group). The same procedure was performed with control mice that had not experienced MS (CONTROL + IRSD and CONTROL + EXPL groups). On PND57-58, all the mice performed the elevated plus maze and the hole-board, social interaction and splash tests. Three weeks after the last episode of defeat, all the mice underwent the CPP procedure with cocaine (1 mg/kg). Irrespective of whether or not MS had taken place, a reduction in open arms measures, dips, and social interaction was observed in mice that experienced IRSD. A higher latency of grooming and acquisition of cocaine-induced CPP were observed only in mice exposed to IRSD alone (CONTROL + IRSD). These results suggest that exposure to a brief episode of stress early in life increases the subsequent resilience of animals to the effects of social stress on vulnerability to cocaine.

Keywords: anxiety-like behaviour, cocaine, conditioned place preference, depression-like behaviour, maternal separation, mice, social defeat, stress inoculation

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1 INTRODUCTION

In spite of cumulative evidence of the potential risks of drug abuse, cocaine is widely consumed among adolescents and young adults (European Monitoring Centre for Drugs and Drug Addiction, 2020). It is clear that biological factors can predispose an individual to cocaine addiction; however, different animal models have demonstrated that environmental factors are also involved (Badiani and Spagnolo, 2013; El Rawas and Saria, 2016; Montagud-Romero et al., 2018; Ahmed et al., 2020).

Among these environmental factors, stress - understood as adversity/negative experiences in life—has been shown to enhance vulnerability to the rewarding effects of cocaine and other drugs of abuse (Aguilar et al., 2013; Rodríguez-Arias et al., 2013; Vannan et al., 2018). Among the different types of stress, social stress is currently the most common, and can be modelled in experimental animals with the chronic/repeated social defeat (SD) paradigm, which is known to have predictive power (Vasconcelos et al., 2015; Patel et al., 2019; Wang et al., 2021; García-Pardo et al., 2022). Several studies have shown an increase in the rewarding effects of cocaine in the conditioned place preference (García-Pardo et al., 2019; Calpe-López et al., 2020; Montagud-Romero et al., 2020) and self-administration (Holly EN. et al., 2016; Rodríguez-Arias et al., 2017) paradigms among animals exposed to SD. In a recent study, we observed that mice exposed to SD displayed anxiety- and depression-like behaviours, social avoidance and greater stress reactivity (Calpe-López et al., 2020).

Another intervention that induces social stress is interference with the maternal-offspring relationship, which has an essential influence on the development of mammals. After birth, pups are vulnerable and the mother carried out important functions, such as protection, warming and feeding, in order to guarantee the physical health and survival of their offspring (Numan and Insel, 2003). Several research works have demonstrated that inadequate maternal care has devastating consequences for the maturation of the central nervous system and mental health of the pups (Yang et al., 2017; Courtiol et al., 2018). In this sense, maternal separation (MS) stress constitutes a critical experience that can induce behavioural alterations and neuropsychiatric disorders in later life (George et al., 2010; Gracia-Rubio et al., 2016; Lukkes et al., 2017; Zhou et al., 2020). Repeated episodes of MS (4–8 h per day, from postnatal day (PND) two to PND16) have been shown to increase the vulnerability of offspring to the rewarding effects of drugs of abuse during adolescence or adulthood (Delavari et al., 2016; Viola et al., 2016; Orso et al., 2017; Castro-Zavala et al., 2020; Castro-Zavala et al., 2021a; Castro-Zavala et al., 2021b; Arenas et al., 2022). Furthermore, different procedures of repeated MS (for 4–8 h per day, from PND2 until PND12-20) enhance the anxiety of adolescent mice in the elevated plus maze (EPM) (Shin et al., 2016; Wang et al., 2017) and in the social preference test (Wang et al., 2017) and induce anhedonia in the saccharin preference test (Wang et al., 2017) and depression-like behaviour in the forced swimming test (He et al., 2020). Moreover, exposure to MS (3 h/day, PND 1–14), though it did not alter the behaviour of mice by itself, was seen to increase the risk of depression-like behaviours in the forced swimming and sucrose preference tests when mice were exposed to an additional restraint stress in late adolescence (PND 42–56) (Han et al., 2019).

However, stress is not necessarily negative, as it can have adaptive properties and induce responses aimed to improve the physical and psychological functioning of the individual (Southwick and Charney, 2012; Brockhurst et al., 2015). In early life, while not of an excessive magnitude, a stressful episode can promote resilience to subsequent stressful experiences later in life (Lyons et al., 2010; Daskalakis et al., 2013; Ashokan et al., 2016). In fact, although resilience is an innate capacity, it is not a stable trait, but rather is a dynamic process that develops throughout a life span (Rutter, 2012; Kalisch et al., 2019) and can be enhanced by different factors (Calpe-López et al., 2022, in press). This could explain why some people rebound after adverse situations while others develop a mental disorder and never recover (Charney, 2004; Yao and Hsieh, 2019). In this sense, exposure to mild or moderate stressors can induce an adaptive stress response in the individual, increasing his/her resilience to the negative effects of future stressful events (Russo et al., 2012; Southwick and Charney, 2012).

Repeated SD has proven itself to be a useful animal model for studying resilience to the negative consequences of social stress and the mechanisms which are involved (Krishnan et al., 2007; Hodes et al., 2014; Henry et al., 2018). A recent study carried out in our laboratory demonstrated that some mice are resilient to the effects of intermittent repeated SD (IRSD) on cocaine reward (Calpe-López et al., 2020). We observed that exposure to IRSD increased the rewarding effects of cocaine in the CPP paradigm, but mice with certain behavioural traits showed resilience to the negative effects of stress (Calpe-López et al., 2020). However, how exposure to an episode of stress in early life affects the subsequent effects of social stress on cocaine reward in later life has not been studied. Thus, the objective of this work was to determine if a brief MS in early life modifies the behavioural response to IRSD in late adolescence and can reverse the potentiating effects of social stress on the rewarding effects of cocaine in adulthood. For this purpose, experimental groups were exposed to MS, IRSD, MS + IRSD or did not undergo stress. Different behavioural tests (EPM, hole-board, social interaction, and splash tests) were employed in order to determine the behavioural effects of both types of stress exposure in late adolescent animals. Three weeks after the last episode of defeat, acquisition of CPP following conditioning with cocaine was evaluated in all the groups. Our hypothesis was that a brief MS in early life can inoculate against the negative effects of subsequent stress and promote resilience to anxiety-, depression- and addiction like symptoms induced by IRSD.

2 MATERIAL AND METHODS

2.1 Animals

Forty-nine male mice of the C57BL/6 strain (born in the Psychology Department Laboratory, University of Valencia, from parents acquired from Charles River, France) and 15 male mice of the OF1 strain (Charles River, France) were used in the present study. They were housed by litter with mother and siblings in plastic cages (25 cm × 25 cm × 14.5 cm). Later, they were weaned and separated from the female mice on PND 21, but remained grouped by litter (3–6 male mice). Mice used as aggressive opponents (OF1) arrived in the laboratory on postnatal day (PND) 21 and were housed individually in plastic cages (23 cm × 32 cm × 20 cm) for three or more weeks before initiation of the experimental procedures in order to induce heightened aggression (Rodríguez-Arias et al., 1998). All mice lived under constant temperature (21°C), a

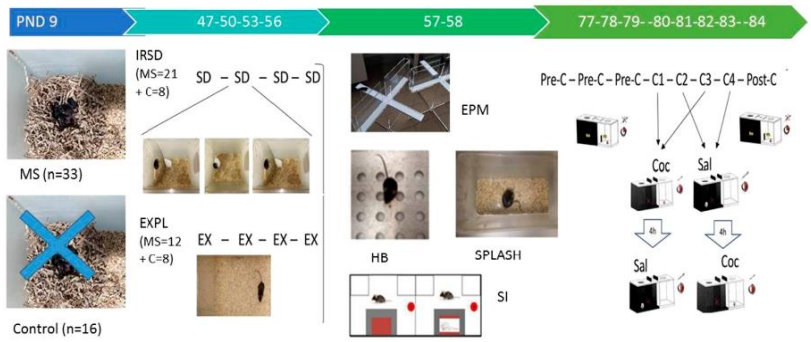

FIGURE 1 | Timeline of experimental procedures. On postnatal day (PND) 9, a group of mice (n = 33) was exposed to a brief stress consisting of 6 h of maternal separation (MS), while a group of other mice did not undergo stress (control, *n* = 16). Subsequently, in late adolescence, mice exposed to MS and controls were divided into two groups in each case. The first MS group was exposed to intermittent repeated social defeat (group 1, MS + IRSD, *n* = 21). On PND 47, 50, 53 and 56, experimental mice were introduced into the cage of an aggressive opponent. Physical contact between them was allowed for only 5 min, during which the experimental mouse experienced social defeat (SD). On the same PND, the other MS group explored (EX) an empty cage (group 2, MS + EXPL, *n* = 12). Control mice without early life stress were divided into the same two groups (group 3, CONTROL + IRSD, *n* = 8; group 4, CONTROL + EXPL, *n* = 8). On PND 57, all mice performed the elevated plus maze (EPM), the hole board (HB) and the social interaction (SI) test. On PND 58, all mice performed the splash test. After an interval of 3 weeks, all mice underwent the conditioned place preference (CPP) paradigm. On PND 77, 78 and 79, mice underwent the pre-conditioning (Pre-C) phase. On PND 80, 81, 82, 83, mice performed four conditioning sessions (C1-C4), in which they received 1 mg/kg of cocaine (Coc) or saline (Sal) before being placed in the drug- or saline-paired compartment, respectively. On PND 84, mice underwent the post-conditioning (Post-C) phase.

reversed 12 h light schedule (on 19:30–07:30) and food and water ad libitum. Before initiation of social defeat or exploration, experimental mice were handled (5 min/day, for 3 days) in order to decrease their stress response to manipulation. All protocols were conducted in compliance with Directive 2010/63/EU and were approved by the Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

2.2 Drugs

For place conditioning, mice received intraperitoneal injections (0.01 ml/g of body weight) of cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) (the same as that used to dissolve the drug). On the basis of previous studies, we employed a dose of 1 mg/kg of cocaine (García- Pardo et al., 2019; Calpe-López et al., 2020).

2.3 Experimental Design

Experimental mice (C57BL/6) were assigned to four groups according to the type of stress experienced in early-life (PND 9) and late adolescence (PND 47, 50, 53 and 56). The first group was exposed to a brief maternal separation and subsequently to four episodes of social defeat (MS + IRSD, *n* = 21); the second group was exposed to MS but did not experience social stress during adolescence (MS + EXPL, *n* = 12); the third group was not exposed to MS but experienced IRSD in late adolescence (CONTROL + IRSD, *n* = 8); and the fourth group was not exposed to MS or IRSD (CONTROL + EXPL, *n* = 8).

The battery of behavioral tests took place on PND 57–58. On PND 57, the mice performed first the EPM, then the hole-board and then the social interaction test, with an interval of 1 h between each test. On PND 58 mice performed the splash test. Subsequently, after a 3-weeks interval, all the mice underwent the CPP procedure (see **Figure 1**). All experiments took place during the dark period (8.30–16.30 h), and mice were introduced into the dimly lit experimental room (different to that of the defeat and exploration procedures) 1 h prior to testing in order to facilitate adaptation.

2.4 Experimental Protocols

2.4.1 Brief Maternal Separation

Repeated experiences of MS (PND2-12 or more, 3–8 h/day), often combined with early weaning (MSEW), constitute an animal model of early-life stress that reproduce the consequences of childhood adversity (George et al., 2010; Vetulani 2013; Bian et al., 2015) and allows researchers to evaluate their impact on the development of depression-like behaviour and on the response of animals to cocaine (Gracia-Rubio et al., 2016; Liu et al., 2018; Vannan et al., 2018; Castro-Zavala et al., 2020). Conversely, by enforcing MS for a short period, we aimed in this study to examine the impact of an acute episode of stress. Newborn mice (*n* = 54) were separated from their mothers for 6 h (9:00–15:00 h) on PND 9 (following a slight modification of the procedure employed in Llorente-Berzal et al., 2013). We selected PND nine for MS because this day marks the end of the neonatal period (PND3-9) and the initiation of postnatal transition (PND9-15) (Fox, 1965). In addition, mice show full retention 24 h after learning on PND 9 (Alleva and D' Udine, 1987). During the separation, the mother was removed and placed in another cage (23 cm × 32 cm × 20 cm) with food

and water access, while the pups remained in their home box. No specific procedure was used to keep the litter warm during this period, as the room temperature in the laboratory was maintained at 21°C and pups have a thick (almost complete) fur on PND 9, which helps thermoregulation. After 6 h, the mother was placed again with her litter. Weaning was carried out on PN21, during which the mice were separated by sex. Only male mice (n = 33) were used for the subsequent experiment. We randomly assigned each litter to the corresponding experimental group (5 litters in the SM + IRSD group, 3 litters in the SM + EXPL group, 2 litters in the CONTROL + IRSD group and 2 litters in the CONTROL + EXPL group). The 5 litters which comprised the 21 male mice of the SM + IRSD group were those in cages 1 (n = 4), 2 (n = 4), 4 (n = 5), 6 (n = 4) and 7 (n = 4). The 3 litters which comprised the 12 male mice of the SM + EXPL group were those in cages 3 (n = 3), 5 (n = 3) and 8 (n = 6). The 2 litters which comprised the eight male mice of the CONTROL + IRSD group were those in cages 9 (n = 4) and 10 (n = 4). The 2 litters which comprised the eight male mice of the CONTROL + EXPL group were those in cages 11 (n = 4) and 12 (n = 4).

2.4.2 Repeated Social Defeat

On PND 47, 50, 53 and 56, the experimental mice (intruders) underwent the RSD procedure, which consisted of four agonistic encounters in which the animal was introduced into the home cage of a conspecific OF1 male mouse that had previously lived in isolation (aggressive opponent). Each encounter lasted for 25 min and consisted of three phases. During the first and last phases, it was protected from attack by a wire mesh wall, which allowed social threats from the aggressive resident (10 min). In the second phase, the wire mesh was removed and confrontation was allowed for 5 min, culminating in the defeat of the experimental mouse (for example, the adoption of an upright submissive position). For more details about the RSD procedure see Calpe-López et al. (2020). The non-defeated animals underwent the same protocol, but without the presence of an aggressive mouse, and simply explored (EXPL) the cage.

2.4.3 Elevated Plus Maze

The effects of stress on anxiety-like behavior were evaluated on PND 57. The EPM consisted of two open and two enclosed arms (30 cm × 5 cm) and was elevated 45 cm above floor level. Mice have a natural aversion to open elevated areas; thus, anxiety is considered to be higher when open arms measurements (time spent and entries) are decreased (Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997). The mice's behaviour in the EPM was video recorded for 5 min and later analysed (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time and percentage of time [(open/open + closed) × 100] spent in the open arms, the number and percentage of open arm entries, time on the centre platform, total distance travelled, number of stretch-attend postures, number of head dips (protected or not) and rearing in the close arms were submitted to statistical analysis. For more details about the EPM apparatus and procedure see Calpe-López et al. (2020).

2.4.4 Hole Board Test

The hole board test, used to evaluate novelty-seeking behavior, was carried out (PND 57) in a square box (28 cm × 28 cm × 20.5 cm) with 16 equidistant holes in the floor (Cibertec SA, Madrid, Spain) and equipped with photocells to detect the number of head-dips performed by the mouse during a 10-min period. For more details about the hole board test see Calpe-López et al. (2020).

2.4.5 Social Interaction Test

On PND 57, the social behaviour of the mice was evaluated in an open field (37 cm × 37 cm × 30 cm), which contained a perforated plexiglass cage (10 cm × 6.5 cm × 30 cm). The mouse was allowed to explore the open field for 10 min on two occasions, separated for 2 min. On the first occasion (object phase), the plexiglass cage was empty. On the second occasion (social phase), a second mouse (OF1 strain) was put into the perforated cage and the experimental mouse was then reintroduced into the open field. In both phases, the time spent by the experimental mouse in the 8 cm area surrounding the perforated cage—considered the interaction zone (IZ) — was automatically registered (Ethovision 2.0, Noldus, Wageningen, Netherlands). An index of social interaction (ISI) was obtained [time spent in the IZ during the social phase/(time spent in the IZ during the social phase + time spent in the IZ during the object phase); Henriques-Alves and Queiroz, 2016]. It is common to use the ISI as the social preference-avoidance index (Krishnan et al., 2007). For more details about the social interaction test see Calpe-López et al. (2020).

2.4.6 Splash Test

The splash test was carried out on PND 58. A 10% sucrose solution was sprayed on the dorsal coat of mice placed in a transparent cage (15 cm × 30 cm × 20 cm) containing bedding, which was designed to encourage grooming behaviour. Mice were recorded for 5 min, and the latency and frequency of grooming were analysed with the aid of a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain) by an observer who was unaware of the treatment administered. Lower frequency of grooming and higher latency to initiate this behaviour are considered to represent depressive-like behaviour (Smolinsky et al., 2009). For more details about the splash test see Calpe-López et al. (2020).

2.4.7 Conditioned Place Preference

Three weeks after the last episode of social defeat, the mice underwent the CPP procedure (PND77-84). Eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a grey central area (13.8 cm long × 31.5 cm wide × 34.5 cm high) were employed. The compartments had different coloured walls (black vs. white) and contrasting floor textures (fine grid vs. wide grid). The position of the animals and their movement between compartments were detected by four infrared light beams in each of the compartments and six in the central area (MONPRE 2Z, Cibertec SA, Madrid, Spain).

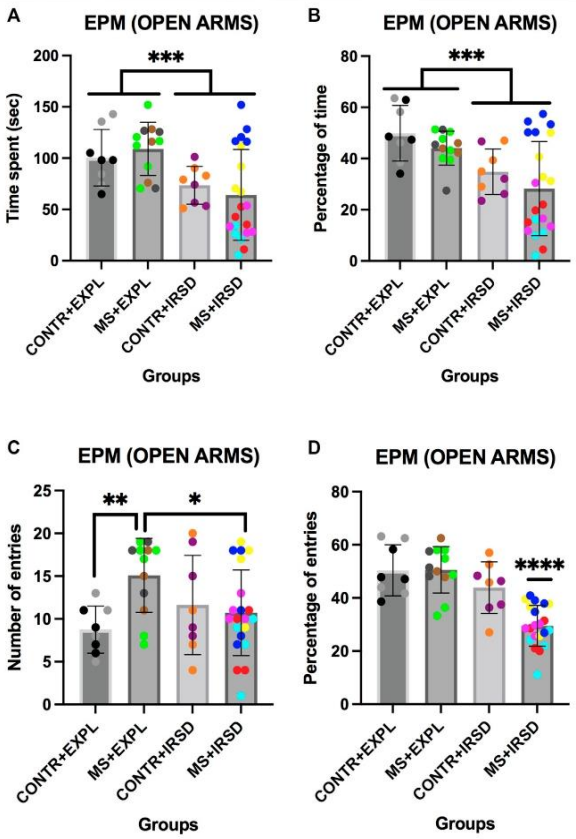

FIGURE 2 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Elevated Plus Maze (Open Arms Measurements). Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) in the late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, $n = 12$) or were exposed to SD (MS + IRSD, $n = 21$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. **(A)** Bars represent the mean (±SD) time spent in the open arms (OA) of the maze for each group. ***$p < 0.002$, significant difference between mice exposed to IRSD and mice exposed to EXPL. **(B)** Bars represent the mean (±SD) percentage of time spent in the OA for each group. ***$p < 0.002$, significant difference between mice exposed to IRSD and mice exposed to EXPL. **(C)** Bars represent the mean (±SD) number of entries into the OA for each group. *$p < 0.05$, significant difference between the groups MS + EXPL and MS + IRSD; **$p < 0.01$, significant difference between the groups CONTROL + EXPL and MS + EXPL (post-hoc comparison of the Interaction Defeat X Separation). **(D)** Bars represent the mean (±SD) percentage of entries into the OA for each group. ****$p < 0.001$, significant difference of the group MS + IRSD with respect to the other groups (post-hoc comparison of the Interaction Defeat X Separation).

The three phases of CPP took place during the dark cycle, and the assignment of the cocaine-paired compartment was carried out following a non-biased design (for more detail, see Maldonado et al., 2007). In summary, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15-min period was recorded, and those with a strong aversion or a preference for a particular compartment (less than 33% or more than 67% of the total time) were removed from the rest of study ($n = 4$). In the second phase (conditioning), lasting 4 days, experimental animals were administered saline and then confined to the vehicle-paired compartment for 30 min. Four hours later, they were injected with 1 mg/kg of cocaine and were immediately confined to the drug-paired

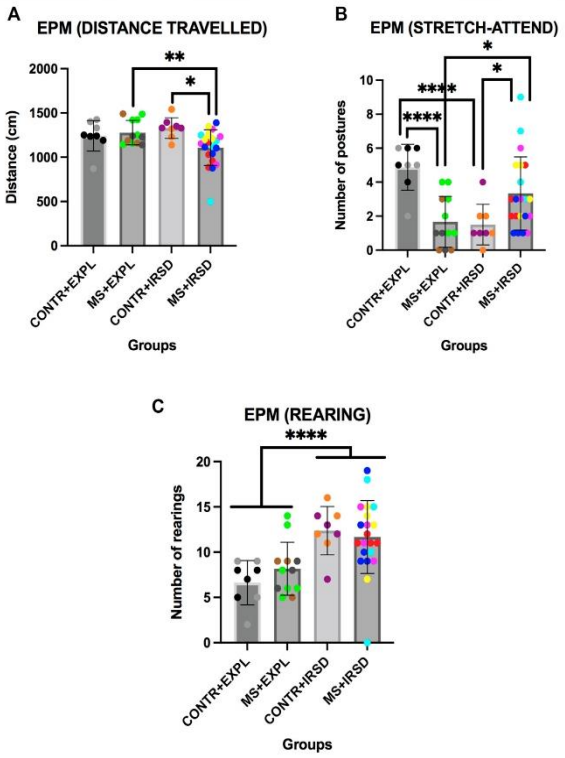

FIGURE 3 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Elevated Plus Maze (Additional Measurements). Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) in the late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, $n = 12$) or were exposed to SD (MS + IRSD, $n = 21$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. **(A)** Bars represent the mean (±SD) distance travelled in the EPM for each group. *$p < 0.05$, significant difference between the groups CONTROL + IRSD and MS + IRSD; **$p < 0.01$, significant difference between the groups MS + IRSD and MS + EXPL (post-hoc comparison of the Interaction Defeat X Separation). **(B)** Bars represent the mean (±SD) number of stretch-attend postures in the EPM for each group. *$p < 0.05$, significant difference between the group MS + IRSD with respect to CONTROL + IRSD and MS + EXPL groups; ****$p < 0.001$, significant difference between the group CONTROL + EXPL with respect to CONTROL + IRSD and MS + EXPL groups (post-hoc comparison of the Interaction Defeat X Separation). **(C)** Bars represent the mean (±SD) number of rearing behaviors in the closed arms of the EPM for each group. ****$p < 0.001$, significant difference between mice exposed to IRSD and mice exposed to EXPL.

compartment for 30 min. The sequence of the injections alternated each day (in the second and fourth conditioning session mice received cocaine first and then saline). During the third phase, or post-conditioning (Post-C), performed 24 h after the last conditioning session, the time spent by the untreated mouse in each compartment during a 15-min period was recorded. A conditioning score for each animal was calculated (time spent in Post-C minus time spent in Pre-C).

2.5 Statistical Analysis

The effects of MS and IRSD were evaluated using a two-way ANOVA with two between-subjects variables - Maternal Separation, with two levels (CONTROL and MS)—and Defeat, with two levels (EXPL and IRSD). Post hoc comparisons were performed with Bonferroni tests. The following behavioural measures were analysed: time, entries, percentage of time and percentage of entries in the open arms of the EPM, time on the centre platform of the EPM, total distance travelled in the EPM,

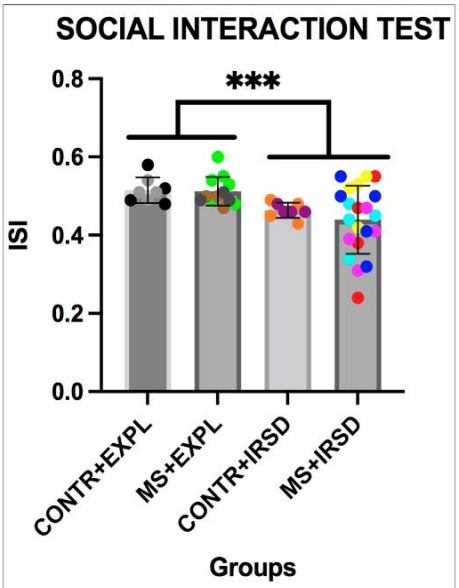

FIGURE 4 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Social Interaction Test. Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) on the late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, $n = 12$) or were exposed to SD (MS + IRSD, n = 21) in late adolescence (PND 47, 50, 53 and 56). The behavior of mice in the social interaction test was evaluated on PND 57. Bars represent the mean (±SD) index of social interaction (ISI) in each group. *$p < 0.002$, significant difference between mice exposed to IRSD and those exposed to EXPL.

number of stretch-attend postures, head dipping (protected or not) and rearing in the close arms of the EPM, number of dips in the hole board test, social interaction index (ISI), latency and frequency of grooming in the splash test, and conditioning score. In order to determine whether there was a relationship among the performances of mice in the different procedures, Pearson correlation tests were carried out. All statistical analyses were performed with the SPSS program.

3 RESULTS

3.1 Effects of Maternal Separation in the EPM

ANOVAs of the time (**Figure 2A**) and percentage of time (**Figure 2B**) spent in the open arms of the EPM revealed a significant effect of the variable Defeat {[F (1, 45) = 11.181, $p < 0.002$] and [F (1, 45) = 13.084, $p < 0.001$], respectively}, while the variable Separation and the Interaction Defeat X Separation were not significant. Mice exposed to IRSD (irrespective of whether they had been exposed or not to MS) spent less time and percentage of time in the open arms than mice exposed to Exploration.

ANOVAs of the number of entries in the open arms of the EPM (**Figure 2C**) revealed a significant effect of the Interaction Defeat X Separation [F (1, 45) = 6.540, $p < 0.05$], while the variables Defeat and Separation were not significant. Post-hoc analysis of the Interaction showed that mice exposed to MS (MS + EXPL group) performed a higher number of entries into the open arms in comparison to control mice (CONTROL + EXP) ($p < 0.01$); in addition, the group exposed to MS and defeat entered the open arms fewer times than mice only exposed to MS (MS + IRSD vs MS + EXPL, $p < 0.05$).

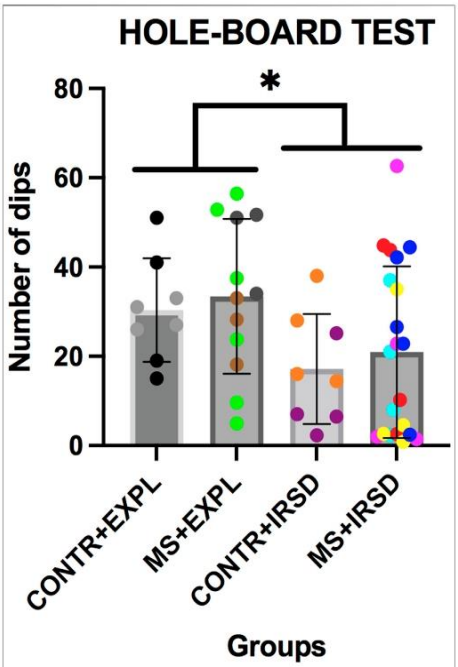

FIGURE 5 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Hole-Board Test. Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, $n = 12$) or were exposed to SD (MS + IRSD, $n = 21$) in late adolescence (PND 47, 50, 53 and 56). The behaviour of mice in the hole board test was evaluated on PND 57. Bars represent the mean (±SD) number of dips in each group. *$p < 0.05$, significant difference between mice exposed to IRSD and mice exposed to EXPL.

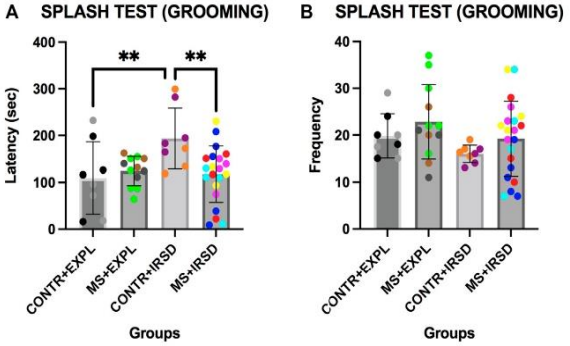

FIGURE 6 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Splash Test. Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, n = 12) or were exposed to SD (MS + IRSD, n = 21) in late adolescence (PND 47, 50, 53 and 56). The behavior of mice in the splash test was evaluated on PND 58. **(A)** Bars represent the mean (±SD) latency of grooming behavior in each group. $^{**}p < 0.01$, significant difference between the group CONTROL + IRSD with respect to CONTROL + EXPL and MS + IRSD groups (post-hoc comparison of the Interaction Defeat X Separation). **(B)** Bars represent the mean (±SD) frequency of grooming in each group.

ANOVAs of the percentage of entries into the open arms of the EPM (**Figure 2D**) revealed a significant effect of the variables Defeat [$F(1, 45) = 27.048$, $p < 0.001$] and Separation [$F(1, 45) = 7.170$, $p < 0.01$], and of the interaction of the two [$F(1, 45) = 7.527$, $p < 0.01$]. Post-hoc analysis of the Interaction showed a lower percentage of entries into the open arms in the group exposed to MS and defeat (MS + IRSD) than mice exposed only to SM (MS + EXPL) or defeat (CONTROL + IRSD) (ps < 0.001).

ANOVA of the total distance travelled in the EPM (**Figure 3A**) revealed significant effects of the Interaction Defeat x Separation [$F(1, 45) = 21{,}415$, $p < 0.001$]. Post-hoc comparison of the Interaction showed that the group SM + IRSD travelled a shorter distance than the SM + EXPL ($p < 0.01$) and CONTROL + IRSD ($p < 0.05$) groups.

ANOVA of the number of stretch-attend postures (**Figure 3B**) revealed significant effects of the Interaction Defeat x Separation [$F(1, 45) = 21{,}415$, $p < 0.001$]. Post-hoc comparison of the Interaction showed that the SM + IRSD group performed a greater number of stretch-attend postures than the CONTROL + IRSD and SM + EXPL groups (ps < 0.05). In addition, the CONTROL + EXPL group performed a greater number of stretch-attend postures than the SM + EXPL and CONTROL + IRSD groups (ps < 0.001).

ANOVA of the number of rearings in the close arms of the EPM (**Figure 3C**) revealed a significant effect of the variable Defeat [$F(1, 45) = 5{,}858$, $p < 0.001$]. A higher number of rearings was observed among mice exposed to defeat in comparison to those exposed to exploration.

ANOVAs of the time spent on the centre platform of the EPM and of head dipping (protected or not) did not reveal significant differences for the variables Defeat or Separation, or for their Interaction (data not shown).

3.2 Effects of Maternal Separation in the Social Interaction Test

ANOVA of data obtained in the social interaction test (**Figure 4**) revealed a significant effect of the variable Defeat [$F(1, 45) = 10.476$, $p < 0.002$], while the variable Separation and the Interaction Defeat X Separation were not significant. A lower ISI was observed among mice exposed to defeat in comparison to those exposed to exploration.

3.3 Effects of Maternal Separation in the Hole-Board Test

ANOVA of the number of dips in the hole-board test (**Figure 5**) revealed a significant effect of the variable Defeat [$F(1, 45) = 5.458$, $p < 0.05$], while the variable Separation and the Interaction Defeat X Separation were not significant. A lower number of dips was observed among mice exposed to defeat in comparison to those exposed to exploration.

3.4 Effects of Maternal Separation in the Splash Test

ANOVA of the latency of grooming in the Splash Test (**Figure 6A**) revealed significant differences for the variables Defeat [$F(1, 45) = 5.641$, $p < 0.05$] and for the Interaction Defeat x Separation [$F(1, 45) = 6.350$, $p < 0.05$]. Post-hoc analysis of the Interaction showed that mice exposed only to defeat (CONTROL + IRSD) displayed a higher latency of grooming than non-defeated mice (CONTROL + EXPL) or mice exposed to both defeat and MS (MS + IRSD) (ps < 0.01). ANOVA of the frequency of grooming (**Figure 6B**) did not reveal significant

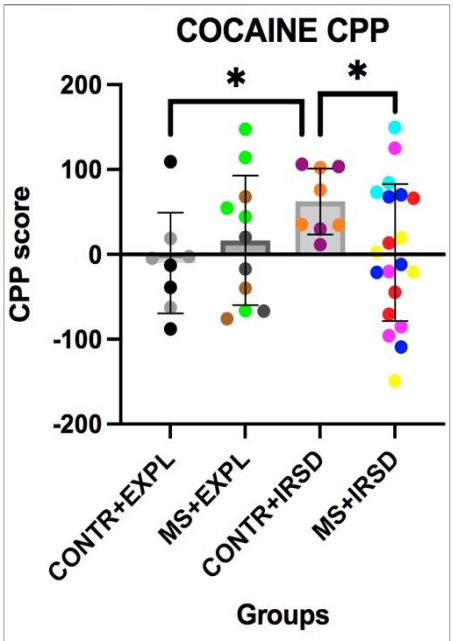

FIGURE 7 | Effects of Maternal Separation (MS) and Intermittent Repeated Social Defeat (IRSD) on the Conditioned Place Preference (CPP) paradigm. Control mice without early life stress explored an empty cage (CONTROL + EXPL, $n = 8$) or were exposed to SD (CONTROL + IRSD, $n = 8$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with early life stress (6 h of MS on PND9) explored an empty cage (MS + EXPL, $n = 12$) or were exposed to SD (MS + IRSD, $n = 21$) in late adolescence (PND 47, 50, 53 and 56). After behavioural tests on PND57-58 and an interval of 3 weeks, mice were conditioned with cocaine (1 mg/kg). Bars represent the mean (±SD) CPP score (in seconds) of each group. *$p < 0.05$, significant difference between the group CONTROL + IRSD with respect to CONTROL + EXPL and MS + IRSD groups (post-hoc comparison of the Interaction Defeat X Separation).

differences for the variables Defeat or Separation or for their Interaction.

3.5 Effects of Maternal Separation in the CPP Paradigm

ANOVA of the conditioning scores (**Figure 7**) revealed a significant effect of the Interaction Defeat X Separation [$F(1, 45) = 3.99$, $p < 0.05$], while the variables Defeat and Separation were not significant. Post-hoc analysis of the Interaction showed that mice exposed only to defeat (CONTROL + RSD) had a higher conditioning score than non-defeated mice (CONTROL + EXPL) or mice exposed to both defeat and MS (MS + RSD) (ps < 0.05).

3.6 Correlations Between Behavioral Measures

Pearson tests revealed the existence of a significant positive correlation between the different measures of the open arms (time, percentage of time, entries and percentage of entries) (see **Table 1**). Some of these open arms measures also correlated with additional measurements evaluated in the EPM such as stretch-attend postures, protected and unprotected head dipping, and distance travelled in the EPM (see **Table 1**). In addition, the ISI positively correlated with the frequency of grooming ($r = 0.297$; $p < 0.05$), with time ($r = 0.480$; $p < 0.001$), percentage of time ($r = 0.522$; $p < 0.001$) and percentage of entries ($r = 0.442$; $p < 0.001$) in the open arms of the EPM, and with the distance travelled in the EPM ($r = 0.352$; $p < 0.05$).

4 DISCUSSION

The present study demonstrates that a brief MS prevents some effects of subsequent IRSD exposure in late adolescent mice, including increased latency of grooming behavior in the splash test and the potentiation of cocaine-induced CPP. However, MS did not modify the social avoidance and anxiety-like behavior induced by social defeat in our animals. Thus, we suggest that

TABLE 1 | Correlations between measures of the elevated plus maze (EPM).

	TOA	EOA	PTOA	PEOA	DISTRAV	PROTHD	UNPROTHD	STR-ATT
TOA	---	0.416	0.934	0,63	0.101	0.086	0.402	-0.327
EOA	**	---	0.348	0.559	0.392	0.284	0.388	-0.284
PTOA	****	*	---	0.712	0.294	0.111	0.426	-0.238
PEOA	****	****	****	---	0.479	0.259	0.374	-0.249
DISTRAV	ns	**	*	***	---	0.268	0.471	-0,4
PROTHD	ns	*	ns	ns	ns	---	0.007	-0,57
UNPROTHD	**	**	**	**	***	ns	---	-0.178
STR-ATT	*	*	ns	ns	ns	ns	ns	---

*Pearson correlations between different measures registered in the EPM. TOA, time spent in the open arms; EOA, number of entries in the open arms; PTOA, percentage of time in the open arms; PEOA, percentage of entries in the open arms; DISTRAV, distance travelled in the EPM; PROTHD, protected head dipping; UNPROTHD, unprotected head dipping; STR-ATT, stretch-attend postures. Upper section of the table: values of the Pearson correlation. Lower section of the table: level of statistically significant correlation between measurements (*p < 0.05, **p < 0.01, ***p < 0.001).*

early stress induced in pups by a brief MS inoculates mainly against the long-term effects of subsequent social stress on vulnerability to cocaine reward.

In accordance with previous studies in our laboratory, we observed that mice exposed to IRSD during late adolescence displayed an increase in the rewarding effects of cocaine in adulthood, since defeated mice acquired CPP after being conditioned with a dose of cocaine, that is, known to be ineffective in inducing place conditioning in non-stressed mice (Montagud-Romero et al., 2017; García-Pardo et al., 2019; Calpe-López et al., 2020). On the other hand, as we expected, the MS procedure employed in our study did not alter the rewarding effects of cocaine. Conversely, other studies have demonstrated that exposure to MS increases the vulnerability of animals to cocaine reward (Matthews et al., 1999; Moffett et al., 2006; Viola et al., 2016; Alves et al., 2020). However, it is important to note that most of the studies in question used a combination of repeated episodes of MS (3 or 8 h per day, from PND2 to PND 12 or later) with early weaning (EW) on PND 14 or 17 (before PND 21, which is the natural moment for weaning) (Kikusui et al., 2004). In fact, MSEW is an animal model of early-life adversity (George et al., 2010; Vetulani 2013; Bian et al., 2015) and permits its impact on cocaine abuse to be evaluated (Liu et al., 2018; Vannan et al., 2018). MSEW causes an impairment of cocaine-induced behavioral sensitization, possibly due to a dysfunction of the dopaminergic system, a potential vulnerability factor for the development of substance use disorders (Gracia-Rubio et al., 2016). In addition, mice exposed to MSEW expressed higher cocaine intake, an enhanced vulnerability to the acquisition of cocaine self-administration, and an incapacity for this behavior to be extinguished (Castro-Zavala et al., 2020; Castro-Zavala et al., 2021a; Castro-Zavala et al., 2021b). In the CPP paradigm, MS (3h/day, from PND2 to PND14-15) also increased vulnerability to cocaine reward in adolescent rats (Alves et al., 2020) and mice (Viola et al., 2016), suggesting that this early life stress subsequently enhances the motivational salience of stimuli associated with cocaine.

The lack of an effect of our MS procedure on cocaine reward and the other behavioral parameters we have evaluated indicated that a single episode of MS (6 h, on PND9) is not a potent stressful event. In fact, our objective was to induce mild stress in early life in order to promote resilience to a subsequent stressful experience later in life, a phenomenon often referred to as stress inoculation (Ashokan et al., 2016). Indeed, the main contribution of our study is that is demonstrates how a brief MS can prevent the long-term effects of IRSD on the rewarding properties of cocaine. In particular, we observed that mice exposed to an episode of MS in early life and to repeated experiences of defeat in late adolescence behaved in the same way as non-stressed mice and did not acquire CPP after conditioning with a low dose (1 mg/kg) of cocaine. Thus, exposure to an episode of MS prevented enhancement of the sensitivity of mice to the rewarding effects of cocaine induced by IRSD. Although the effects of MS on the subsequent influence of stress on cocaine reward has not yet been evaluated, our results are in line with those of some studies which have demonstrated that neonatal stress procedures, including MS, can reduce the rewarding effects of drugs of abuse such as morphine (Boasen et al., 2009), MDMA (Llorente-Berzal et al., 2013) and cocaine (Hays et al., 2012). In addition, inoculation against stress early in life by means of disrupting dam-pup interactions (MS or limited bedding) was found to increase subsequent resilience to the effects of chronic SD stress on several physiological and behavioral parameters (Hsiao et al., 2016; Qin et al., 2019).

The protective effects of our MS procedure on the short-term effects of IRSD were less consistent. One or 2 days after the last episode of IRSD, late adolescent mice showed a reduction in all measurements related to the open arms of the EPM (considered to represent anxiety-like behavior; Campos et al., 2013), a reduced number of dips in the hole board (indicative of low novelty-seeking behaviour; Vidal-Infer et al., 2012), a deficit in social interaction, and a higher latency of grooming in the splash test (considered to represent depression-like behavior; Butelman et al., 2019). These results are in accordance with those of previous studies performed in our laboratory in which we observed that IRSD increased anxiety-like behavior in the EPM (García-Pardo et al., 2015; Calpe-López, et al., 2020), reduced social interaction (García-Pardo et al., 2015; Calpe-López, et al., 2020), induced social subordination (Rodríguez-Arias et al., 2016) and increased depression-like behavior (reduction in the frequency of grooming in the splash test) (Rodríguez-Arias et al., 2016; Calpe-López, et al., 2020). Conversely, our MS procedure did not alter the behavior of mice in any of the tests performed. These results contrast with data showing that MS increases social avoidance and induces anxiety- and depression-like behavior (Bian et al., 2015; Rana et al., 2015; Shin et al., 2016; Alves et al., 2020; He et al., 2020); nevertheless, it should be taken into account that these studies included repeated experiences of MS, while we used a single MS episode in order to induce a mild stress. In this line, a recent study has demonstrated that prolonged MS (3h/day, from PND1-21), but not short MS (15 min/day, from PND1-21), increases susceptibility to depression-like behavior when mice are exposed to chronic unpredictable mild stress in adulthood (Bian et al., 2021). In the present study, mice exposed to a brief MS became resilient to the depression-like behavior induced by exposure to IRSD in late adolescence (as indicated by changes in the latency of grooming). This result is especially important, as it underlines a close link between depression and cocaine abuse (Filip et al., 2013; Xu et al., 2020). In this sense, a recent study in our laboratory showed that resilience against the depression-like behavior (reduction in the frequency of grooming) observed a short time after IRSD is a behavioral trait related with subsequent resilience against the long-term effects of IRSD on cocaine reward (Calpe-López et al., 2020).

However, our MS protocol did not prevent other effects of IRSD, including anxiety-like behavior in the EPM, the reduced number of dips in the hole-board, and a deficit in social interaction. Some of these effects could also be related with the behavioral profile of mice that were resilient to the effects of IRSD on cocaine reward. In our previous study we observed that defeated mice that spent a lower percentage of time in the open arms of the EPM and performed a lower number of dips in

the hole-board a short time after IRSD (animals exhibiting a greater concern for potential dangers in novel environments) are resilient against the potentiation of cocaine CPP induced by IRSD; conversely, defeated mice that displayed mild anxiety-like behavior and marked novelty-seeking behavior a short time after defeat were more vulnerable to the long-term effects of IRSD and developed CPP after conditioning with a low dose of cocaine (Calpe-López et al., 2020). In the present study, MS did not prevent the effects of IRSD in the EPM, since mice exposed to MS + IRSD exhibited a similar profile to mice exposed only to defeat (CONTROL + IRSD). Indeed, mice exposed to MS + IRSD showed a decrease in the percentage of entries in the open arms of the EPM and a reduction in the distance travelled in the EPM, neither of which were observed among mice exposed to IRSD or MS alone. On the other hand, mice exposed to MS + IRSD performed a greater number of stretch-attend postures than mice in the CONTROL + IRSD and MS + EXPL groups, but the MS + IRSD group was the only one that did not differ from non-stressed mice (CONTROL + EXPL group). Although an increase in stretch-attend postures has been interpreted as representing enhanced anxiety (Grewal et al., 1997), we observed higher values among mice in the CONTROL + EXPL group, which raises doubts about the true meaning of this measure. Stretch-attend postures in the EPM can be interpreted as a measure of risk behavior which occurs when the animal experiences an exploratory-anxiety conflict (Holly KS. et al., 2016). There was a positive correlation amongst all measurements in the open arms (time, entries, percentage of time, percentage of entries) and with unprotected head dipping, suggesting that this latter measure is also indicative of lower anxiety. Similarly, the distance travelled correlated positively with number of entries, percentage of entries and percentage of time in the open arms. All these measurements were higher in non-stressed mice than in defeated animals, thus indicating anxiety-like behavior irrespective of whether or not there was exposure to MS. Conversely, there was a negative correlation between the number of stretch-attend postures and the number of entries and time spent in the open arms, but not with the percentages of these measures. These results may simply indicate that mice perform stretch-attend postures more frequently when they are in closed arms than when they are in open arms.

The fact that MS did not ameliorate the social interaction deficit induced by IRSD also contrasts with our previous study in which resilient defeated mice that did not develop cocaine CPP were also characterized by a lack of social avoidance (Calpe-López et al., 2020). From our point of view, the most plausible explanation is that a more pronounced early-life stress is necessary to induce inoculation against the short-term effects of IRSD on the EPM and social interaction test. In support of this hypothesis, it has been observed that repeated MS (1 h/day, from PND 3–21) alleviates the increased anxiety-like behavior induced by chronic SD stress in adulthood (Qin et al., 2019). Similarly, fragmented dam-pup interactions during PND2-9 (by limiting bedding and nesting material in the cage) was seen to reduce the social interaction deficit induced by chronic SD stress (Hsiao et al., 2016). While MSEW induced a depression phenotype and increased cocaine abuse (Liu et al., 2018; Vannan et al., 2018), we observed that a mild MS stress prevented the depression-like behavior and potentiation of cocaine reward induced by IRSD, though not enough to counteract other effects of IRSD. Future studies need to determine the level of MS that induces positive effects and effectively reverses all the effects of subsequent stress exposure. Age and sex could be mediating factors in the inoculation against stress by MS, since adolescence is a period of extreme vulnerability to the effects of drugs of abuse and the development of mental disorders (Dow-Edwards et al., 2019), and there are distinctive sex differences in these disorders, including substance use disorders (Becker, 2016; Li et al., 2017). Thus, it could be relevant to evaluate the effects of MS in female mice exposed to vicarious social defeat stress. Furthermore, the genetic predisposition of subjects to low or high emotional reactivity may be an important factor in determining the positive or negative effects of MS. Rats with a high novelty response and low anxiety/depression levels have been found to be resilient to the negative physiological effects of MS stress (3 h/day, from PND1-14) (Clinton et al., 2014). The same protocol of MS induced social avoidance and anxiety-, and depressive-like behaviors in Wistar rats, but had the opposite effects in Wistar-Kyoto rats, an animal model of comorbid depression and anxiety (Rana et al., 2015). The present study has other limitations. First, we have evaluated only the effects of MS on the CPP induced by a low dose of cocaine. This single-dose experiment provided limited information, and so a complete dose-response study would need to be performed in order to draw solid conclusions about the effects of MS on cocaine reward. Second, the design of our study can induce litter effects; in other words, mice from the same litter are phenotypically more similar than mice from different litters. Litter effects account for an elevated percentage of variability and can mask the true effects of an experimental treatment. Thus, the impact of litter-to-litter variability should be controlled and minimized in order to enhance the rigor and reproducibility of the results observed in this study.

Our results suggest that inoculation against stress early in life through a brief episode of MS increases subsequent resilience to some of the negative effects of IRSD stress, since it prevents the development of depression-like behavior in mice defeated in late adolescence and long-term enhancement of their sensitivity to cocaine reward in adulthood. In terms of the mechanisms underlying such adaptive changes, we hypothesize that the glutamatergic system and the hypothalamus pituitary adrenal (HPA) axis are involved. A moderate MS that prevented the increase in anxiety-like behavior induced by chronic SD stress in adulthood was also seen to prevent the hyperactivity of glutamatergic transmission in the basolateral amygdala induced by this kind of stress (Qin et al., 2019). In addition, mice exposed to moderate early life stress show less social interaction deficits after chronic SD stress (Hsiao et al., 2016), and exhibit a significant decrease in the corticosterone response to a subsequent stressful event (Plotsky and Meaney, 1993; Hsiao et al., 2016). No studies have been performed about the inoculating effect of MS on the subsequent response of stressed animals to drugs of abuse. However, studies of the mechanisms than underlie the effects of more stressful protocols of MS on the rewarding properties of cocaine have

revealed that MS modifies the activity of AMPA and NMDA receptors in structures of the brain reward circuit and other areas involved in the learning of cocaine-cue association (Ganguly et al., 2019; Castro-Zavala et al., 2020; Castro-Zavala et al., 2021b). MSEW was seen to enhance glutamatergic function in the nucleus accumbens and increase excitability of ventral tegmental area DA neurons (Castro-Zavala, et al., 2021a). Moreover, the impairment in reward function induced by MS was reversed by blocking glutamate signaling during adolescence (O'Connor et al., 2015). Additionally, a link between MS and cocaine reward and levels of tumor necrosis factor (Ganguly et al., 2019), brain-derived neurotrophic factor (BDNF, Viola et al., 2016) or TrkB receptors (Orso et al., 2017) has been demonstrated. We hypothesize that the protective effects of a brief MS on the subsequent potentiation of cocaine reward induced by IRSD is also mediated by the glutamatergic system and modifications of both the HPA system and different signaling pathways. In previous studies in our laboratory, we observed that social defeat decreased the expression of several subunits of NMDA and AMPA receptors (García-Pardo et al., 2019), and that the antagonism of NMDA receptors before each episode of defeat prevented the potentiation of cocaine CPP induced by IRSD. The same effect has been observed with the antagonism of CRF receptors (Ferrer-Pérez et al., 2018). Dopaminergic pathways, BDNF signaling and TrkB receptors also play a role in the effects of IRSD on cocaine reward (Montagud-Romero et al., 2017). Future research should attempt to unravel the cellular and molecular mechanisms underlying the protective effect of brief or moderate protocols of MS on the negative consequences of social defeat stress. In addition, the mother's reactions when returned to her litter may contribute to the inoculation against stress induced by brief MS. The potential impact of mother/pup interactions before and after MS on the outcomes observed in the offspring should be evaluated by future research. Such studies could help to develop new intervention approaches for the prevention of stress-related disorders and new therapeutic strategies to treat vulnerable individuals at risk of developing a drug use disorder following stressful experiences.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

AUTHOR CONTRIBUTIONS

MAA and MPG-P contributed conception and design of the study; CC-L and MAM-C performed the experiments, organized the databases and performed the statistical analyses; CC-L and MAM-C wrote some sections of the manuscript, CC-L and MPG-P wrote the complete first draft of the manuscript and MAA wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Experimental Study 4

Inoculating to the Behavioral Effects of Intermittent Repeated Social Defeat in Adolescence Male Mice.

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(In preparation)

ABSTRACT

Although exposure to intermittent repeated social defeat (IRSD) increases the vulnerability of mice to the rewarding effects of cocaine in the conditioned place preference (CPP) paradigm, some defeated mice are resilient to stress and do not develop CPP. In order to prevent CUD, it is important to discover strategies that can enhance resilience. According to the stress inoculation hypothesis, exposing subjects to a situation of low stress could decrease their maladaptive responses to future stress experiences. In this line, the aim of the present study was to evaluate whether the exposure to an acute stress during the early adolescence were effective to increase resilience to the effects of IRSD later in life. To reach this goal, on PND 27 mice were exposed to an acute immobilization (IMM, restrain stress), a social defeat episode (SD, social stress), a vicarious social defeat (visualization of the social defeat of an animal of the same strain (VSD, emotional stress) or did not experience stress. On late adolescence (PND 47, 50, 53 and 56), half animals of each group were exposed to IRSD in the cage of an aggressive opponent (Control+IRSD, IMM+IRSD, SD+IRSD and VSD+IRSD groups) while the other half only performed the exploration of a empty cage (Control+EXPL, IMM+EXPL, SD+EXPL and VSD+EXPL groups). Then, all the animals carried out different behavioral tests, the Elevated Plus Maze (EPM), Hole-Board and Social Interaction Test on PND 57, and the Tail Suspension and Splash tests on PND 58. Three weeks later, all mice performed the CPP paradigm with a low dose of cocaine (1 mg/kg) (PND 77-84). All protocols of stress on early adolescence induced resilience to the potentiation of cocaine CPP induced by exposure to IRSD on late adolescence and to the increase in the latency of entry into the open arms of the EPM. In addition, exposure to an acute social defeat on PND 27 reversed IRSD-

induced social avoidance while exposure to immobilization on PND 27 reversed all the effects of IRSD on the EPM measures. Taken together, our data demonstrate that exposure to an acute experience of stress in early adolescence can enhance resilience of mice to the long-term consequences of IRSD on cocaine reward. However, the development of resilience to several short-term effects of stress depend on the type of stress experienced during early adolescence. In sum, our results support the hypothesis of stress inoculation in adolescent male mice.

Keywords: resilience, acute social defeat, defeat visualization, immobilization, conditioned place preference, stress inoculation, cocaine, male mice

Abbreviations: *CPP, conditioned place preference; EPM, elevated plus maze; FG, frequency of grooming; ISI, index of social interaction; NS, novelty-seeking; PND, post-natal day; RSD, repeated social defeat; TI, time of immobility; TST, tail suspension test; %TOA, percentage of time in open arms.*

INTRODUCTION

Stress exposure has a long-term impact on motivated behavior and can exacerbate preexistent vulnerabilities for the development of a substance use disorder (SUD) (Engeln et al., 2021). Several models have been developed to examine how stressful experiences influences drug rewards. The scientific literature reveals that social stress exposure in animal models induces increased drug reinforcement responses and facilitate the reinstatement of drug seeking after periods of abstinence in the conditioned place preference (CPP) and the drug self-administration paradigms (Aguilar et al., 2013;

Montagud-Romero et al., 2018; Rodriguez-Arias et al., 2016, 2017). In previous studies of our laboratory, we have demonstrated that stress-induced by intermittent repeated social defeat (IRSD) enhances the rewarding effects of cocaine in the CPP paradigm in mice (García-Pardo et al., 2019; Calpe-López et al., 2020). However, we have also observed individual differences in response to IRSD; in fact, we can distinguish two subpopulations of mice. Some animals develop depression-like symptoms and increased sensitivity to cocaine reward (susceptible or vulnerable animals), while others show a clear resistance to at least some of these maladaptive sequelae of stress (resilient animals) (Calpe-López et al., 2020). The phenomenon of resilience, understood as the ability of subjects to overcome the negative effects of stress, has been the subject of growing interest in recent years and has implicated in a paradigm shift in the fields of medicine and psychology, as it focuses on factors that maintain health and promote well-being rather than factors of vulnerability to disease. Resilience could explain why not all individuals who undergo stressful experiences become addicted to drugs of abuse. Using the chronic social defeat stress (CSDS) model, Krishnan et al. (2007) demonstrated that only mice characterized as susceptible (mice that displayed social avoidance after RSD exposure) developed cocaine-induced CPP. We have also previously demonstrated that mice that showed less submission during defeat episodes, and a behavioral profile short-term after IRSD characterized by lower percentage of time in the open arms of the elevated plus maze (EPM), low novelty-seeking, high social interaction, greater immobility in the tail suspension test (TST) and a higher frequency of grooming were resilient to the long-term effects of IRSD on cocaine reward since they behaved like controls and did not develop CPP (Calpe-López et al., 2020).

A novel approach to reduce the incidence of substance use and other stress-related disorders is to promote resilience to stress using environmental resources. For example, we have demonstrated that exposure to physical exercise (Voluntary Wheel Running, VWR) during adolescence subsequently blocks the negative consequences of stress induced by IRSD in the EPM, splash test and CPP, since the defeated mice previously exposed to VWR did not display anxiety- or depression-like effects or the potentiation of cocaine reward observed in mice exposed only to IRSD (Calpe-López et al., 2022a). In the same line, another study has shown that **a** brief 9-day cognitive training protocol in young adulthood may promote long-term resilience to drug-seeking behavior, since after an interval of 4 weeks mice that have received the cognitive training showed a reduced maintenance of cocaine CPP in comparison to mice kept in standard housing for the same period that did not extinguish CPP (Boivin et al., 2015). Results of these studies suggest that while environmental stress increase vulnerability to drugs of abuse, positive experiences may build resilience to future drug challenges (Boivin et al., 2015).

Another way to increase resilience could be stress inoculation, a phenomenon which consists of exposing subjects to a situation of low stress level and if possible, controllable by the individual, decreasing their maladaptive response to future stress exposures. In this sense, we have demonstrated that exposure to a brief episode of stress early in life (6 h of maternal separation on post-natal day 9) increases the subsequent resilience of animals to some effects of IRSD, such as the depression-like symptoms in the splash test (higher latency of grooming) or the potentiation of cocaine reward (Calpe-López et al., 2022a). However, other effects of IRSD, including the anxiety-like behavior in the EPM and social avoidance were not prevented by

maternal separation (Calpe-López et al., 2022b). Hays et al. (2012) also showed that neonatal stress (including 8 h of maternal separation on post-natal days 5-9) decreased the cocaine-CPP response and increased hippocampal neurogenesis in adult mice that, according to the authors, have been desensitized by early life stress and showed a reduced stress responsiveness during CPP test. Similarly, interference with natural dam-pup interactions during postnatal days 2-9 (a model of early life stress) reduced the social avoidance deficits induced by CSDS, enhanced stress coping behaviors in the TST and forced swimming test and decreased corticosterone response to acute restraint stress (Hsiao et al., 2016). These results support the stress inoculation hypothesis whereby a stressful experience in early life could increase the capacity of subjects to face stressors later in life.

The hypothesis of stress inoculation has also been evaluated in young adult mice (Ayash et al., 2020; Brockhurst et al., 2015). To induce stress inoculation the experimental mouse was introduced into the cages of an aggressive mice, with a mesh wall between the two, for 15 min during 11 sessions. Inoculation training sessions acutely increased plasma corticosterone levels but subsequently reduced corticosterone responses to repeated restraint (Brockhurst et al., 2015). In addition, stress inoculation reduced immobility in the TST, decreased freezing in the open field and decreased novel-object exploration latencies in mice exposed to repeated restraint (Brockhurst et al., 2015). Similarly, Ayash et al. (2020) reported that, compared to non-inoculated control mice, stress-inoculated mice showed more active defense behavior in an acute social defeat encounter, higher sociability, better extinction of conditioned fear, lower immobility in the tail suspension test and reduced defecation in an open-field test. These results

suggest that stress inoculation protects against different types of stressors and could be used to improve coping strategies and build resilience.

Although adolescence is a critical developmental phase with enhanced susceptibility to stress (Tschetter et al., 2022), no previous studies have evaluated the effects of inducing stress inoculation in adolescent animals on their responsivity to stress later in life. Thus, the aim of the present study was to assess the stress inoculation hypothesis using different procedures to induce stress in early adolescent mice, such as exposure to an immobilization stress for 10 min (physical stress), exposure to an acute social defeat (social stress) or exposure to a vicarious social defeat through the visualization of the social defeat of another animal (emotional stress). Subsequently, we examined whether these environmental manipulations can enhance the resilience of animals to the effects of IRSD during late adolescence in the EPM, splash test, social interaction test, hole-board and TST. In addition, we assessed if stress exposure during early adolescence can prevent the long-term effects of IRSD on the rewarding effects of cocaine in the CPP paradigm in adult mice.

MATERIAL AND METHODS

Subjects

A total number of 98 male mice of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were delivered to our laboratory at 21 days of age and 42 days of age respectively. They were housed for 6 days before initiation of the experimental procedures. Experimental mice (C57BL/6) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). Mice used as aggressive opponents (OF1) were

individually housed in plastic cages (23 × 32 × 20 cm) in order to induce heightened aggression (Rodríguez-Arias et al., 1998). To reduce their stress levels in response to experimental manipulations, grouped mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures. All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available ad libitum, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocol was approved by the Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

Drugs

Animals were injected intraperitoneally with 1 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The physiological saline was also used to dissolve the cocaine. The dose of cocaine was selected on the basis of previous studies (Rodríguez-Arias et al., 2017; García-Pardo et al., 2019).

Experimental Design

After an adaptation period, on PND 27 the experimental mice (C57BL/6) were assigned to one of three different stress-protocol groups (Immobilization, Social Defeat or Vicarious Social Defeat) or to a control group without stress. On PND 47, the mice of each protocol of stress were assigned to one of two groups: one group was subsequently exposed to four

episodes of social defeat (IRSD on PND 47, 50, 53 and 56) in the cage of a resident mice (IMM+IRSD, n=15; SD+IRSD, n=15; VSD+IRSD, n=15), while the other group did not undergo stress and explored an empty cage on the same days (IMM+EXPL, n=12; SD+EXPL, n=13; VSD+EXPL, n=12). Control mice, which did not undergo any stress protocol, were also assigned to two groups: one group was exposed to IRSD on PND 47, 50, 53 and 56 (Control+IRSD, n=8), and the other group explored an empty cage on the same days (Control+EXPL, n=8).

On PND 57–58, all mice underwent a series of behavioral tests: elevated plus maze (EPM), hole-board (HB), social interaction (SI), splash (SH) and tail suspension (TS) tests. On PND 57, the mice performed first the EPM, followed by the HB and then the SI test, with an interval of 1 hour between each test. On PND 58, mice performed the SH test and, after an interval of 1 hour, the TS test. The order of tests was based on a previous study carried out in our laboratory. Afterwards, all mice were housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (PND 77-84) in order to evaluate the long-term effects of the experimental manipulations undergone during adolescence on cocaine reward in the adult mice (see Fig. 1b). All experiments took place during the dark period (8.30h–16.30h) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing.

Experimental Protocols

1. Protocols of inoculation.

1.1. Protocol 1: Immobilization

To evaluate the effects of an acute episode of restrain-induced stress (Patel et al. 2005; Lu et al. 2003), animals were submitted to immobilization for 10 min on PND 27 (See Fig. 1a). To induce restraint, when mice spontaneously passed into a cylindrical glass tube (4 cm in diameter and 10 cm in length, with holes 0.5 cm in diameter to permit respiration), two test tubes 0.5 cm in diameter were carefully introduced underneath the animal thus reducing the size of the tube to 3 cm so that it was impossible for the animal to turn (Ribeiro Do Couto et al., 2006).

1.2. Protocol 2: Acute Social Defeat

In this paradigm the experimental animal suffers an acute experience of social defeat on PND 27 in an agonistic encounter with a conspecific aggressive animal which had been previously isolated for at least one month (Fujii et al., 2019; Thomas et al., 2021) (See Fig. 1a). Agonistic encounters are performed in a neutral transparent plastic cage (23 x 13.5 x 13 cm), different of the home cage of the experimental or the aggressive animal. First, both animals are placed in the neutral cage separated by a transparent plastic barrier during 1 min. Then, this barrier is removed and the physical interaction between them is allowed for 10 min. In response to the aggressive behaviors of the opponent, experimental animal (group-housed and without fighting experience) exhibited avoidance/flee and defensive/submissive behaviors. The criteria used to define an animal as defeated is a specific posture,

characterized by an upright position, limp forepaws, upwardly angled head, and retracted ears.

1.3. Protocol 3: Vicarious Social Defeat (VSD) Stress

In this paradigm of social defeat, the experimental animals visualize a short experience of social defeat between a conspecific-defeated mouse and an aggressive-opponent mouse (5 min). The VSD stress is a novel paradigm capable of inducing emotional stress by isolating physical stress/confrontation in mice (Sial et al., 2016). In this model, male mice exhibit depressive-like behaviors after witnessing the defeat bout of a same-sex conspecific, which resembles the behavioral profile of physically stressed mice (Iñiguez et al., 2014; Hodes et al., 2014).

We used a neutral transparent plastic cage (29 x 60 x 35 cm), different of the home cage of the animals, for the agonistic encounters. We also constructed two meshes made of metal grid (9 x 60 x 35 cm), each of them consisting of six compartments (9 x 10 cm) to separate the experimental mice of the defeated and opponent mice during the social defeat encounters.

Regarding the method employed, we triggered an agonistic encounter with the result of VSD stress for the experimental mouse. The agonistic encounter between the defeated mouse and the aggressive opponent takes place in the central corridor of the neutral transparent plastic cage described above, so that all experimental mice can smell and observe the defeat through the metal grid.

First, the experimental animals are placed into the wire mesh separated compartments (six in each side of the plastic cage). Then, an OF1 aggressive male mouse is placed in the central corridor so it can freely explore this intermediate space during 3 minutes. Immediately, a C57 male mouse is

introduced into the central corridor and the agonistic encounter takes place during 5 minutes, while the experimental mice are allow only a vicarious experience (i.e., visual, olfactory, auditory) of the physical bout. (See Fig. 1a)

2. Intermittent Repeated Social Defeat (IRSD)

The IRSD procedure consisted of four encounters - separated by intervals of 72 h (PND 27, 30, 33 and 36) - with a conspecific isolated mouse (OF1), which resulted in the defeat of the experimental animal. Each encounter lasted for 25 min and consisted of three phases, which began by introducing the experimental animal (intruder) into the home cage of the aggressive opponent (resident) for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall, which allowed social interaction and threats from the aggressive male resident. The wire mesh was then removed from the cage and confrontation between the two mice was allowed for 5 min. In the third phase, 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. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. 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; Ribeiro Do Couto et al., 2006). All experimental mice displayed defeat, given that they all faced resident mice with high levels of aggression. The first and fourth agonistic encounters were videotaped and evaluated by an observer who was blind to the treatment (Brain et al., 1989) using a computerized system (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent in avoidance/flee

and defense/submission by the experimental mice and the time spent in threat and attack by the resident aggressive mice were measured, as were the latencies of these behaviors. The control (non-stressed) group underwent the same protocol, without the presence of a ‘‘resident’’ mouse in the cage.

3. Elevated Plus Maze (EPM)

The effects of RSD on anxiety were evaluated on PND 37 using the EPM paradigm. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open-space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and the measurements in the closed arms are lower, and vice versa (Rodgers and Dalvi, 1997; Rodgers and Johnson, 1995). Moreover, the total number of entries into the arms are regarded as locomotor activity scores (Campos et al., 2013; Valzachi et al., 2013). At the beginning of each trial, subjects were placed on the central platform facing an open arm and were allowed to explore it for 5 min. The maze was cleaned with a 7% alcohol swab after each test, and the device was allowed to dry completely. The behavior of the mice was video recorded and

later analyzed by an investigator blind to the experimental conditions, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The measures recorded during the test period were frequency of entries and time spent in each section of the apparatus (open arms, closed arms and central platform). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: latency to first enter the open arms; time and percentage of time [(open/open + closed) × 100] spent in the open arms; number and percentage of open arm entries; and total entries into the arms.

4. Hole-Board Test

The mice's novelty-seeking was evaluated in the hole board test 24 h after the last defeat or exploration (PND 37). This test was carried out in a square box (28 × 28 × 20.5 cm) with transparent Plexiglas walls and 16 equidistant holes of 3 cm in diameter on the floor (CIBERTEC SA, Madrid, Spain). Photocells below the surface of the holes detected the number of times that a mouse performed a head-dip. At the beginning of the test, mice were placed in the same corner of the box and were allowed to freely explore the apparatus for 10 min. The latency to the first dip and the frequency of dips were automatically recorded by the apparatus.

5. Social Interaction Test

Twenty-four hours after the last defeat or exploration (PND 37), the social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated plexiglass cage (10 × 6.5 × 30 cm) was placed in the middle of

one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions. The first time (object phase) the perforated plexiglass cage was empty. After 10 min of exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was confined to the perforated cage (to safeguard the experimental mouse from attack) and the experimental mouse was reintroduced into the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the RSD episodes). In both phases, the time spent in the 8 cm area surrounding the perforated cage—the interaction zone—was registered and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An index of social interaction (ISI) was obtained [time spent in the interaction zone during the social phase/(time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase); Henriques-Alves and Queiroz, 2016]. The ISI is commonly used as the social preference-avoidance index (Krishnan et al., 2007).

6. Tail Suspension Test (TST)

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair (Pollak et al., 2010). It is based on the observation that, after initial escape-oriented movements, rodents develop an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung by their tail in an uncontrollable fashion

(Cryan et al., 2005). This has been used as a measure of behavioral depression because, when antidepressant treatments are given prior to the test, the subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle (Pollak et al., 2010). Forty-eight hours after the last defeat or exploration (PND 38), we investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the TST. In accordance with the protocol described by Vaugeois et al. (1997), mice were suspended by the tail, using adhesive tape, from a hook connected to a strain gauge that recorded their movements during a 6-min test period. The behavior displayed by the mice was video recorded and later analyzed by an observer who was blind to the treatment received by the animal, using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameters considered for the statistical analyses were the total time spent immobile and the latency to become immobile.

7. Splash Test

The splash test was carried out on PND 38 and consisted of spraying a 10% sucrose solution on the dorsal coat of a mouse placed in a transparent cage (15 × 30 × 20 cm) with regular bedding to stimulate grooming behavior. The behavior of the mice was videotaped for 5 min and later analyzed using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain) by an observer blind to the treatment received by the animal. The latency to the first grooming, the time spent engaged in this behavior and its frequency were recorded. An increase in latency to grooming and a decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior (Smolinsky et al., 2009).

8. Conditioned Place Preference (CPP)

Three weeks after the last episode of social defeat (PND 57), the animals carried out the CPP procedure. For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 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 recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain). The CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for detailed explanations of the procedure, see Maldonado et al., 2007). In brief, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15-min period was recorded. Animals showing a strong unconditioned aversion or a preference for a given compartment were excluded from the study (n=2). In the second phase (conditioning), which lasted for 4 days, experimental animals received saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, were injected with 1 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 min. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment during a 15-min period was recorded.

Statistical Analysis

The behavioral effects of each protocol of stress inoculation and IRSD were evaluated using a two-way ANOVA with two between-subjects variables; Inoculation, with two levels (Control and IMM, Control and SD or Control and VSD), and Defeat, with two levels (EXPL and IRSD). Post hoc comparisons were performed with Bonferroni tests, which allow multiple hypotheses to be tested simultaneously, thus limiting the type I error rate without increasing the probability of a type II error occurring. The effects of each protocol of stress inoculation and IRSD on the CPP paradigm were evaluated using a three-way ANOVA with the two between-subjects variables described above and a within-subjects variable; Days, with two levels (Pre-C and Post-C). Post hoc comparisons were performed with Bonferroni tests. All statistical analyses were carried out with the SPSS program.

RESULTS

Effects of the exposure to an acute immobilization on early adolescence

The ANOVA of the number of entries into the open arms of the EPM revealed that the variable Inoculation [$F(1,39)=17.518$; $p<0.001$], Defeat [$F(1,39)=6.413$; $p<0.05$], and the Interaction of the variables Inoculation X Defeat [$F(1,39)=8.655$; $p<0.01$] were significant (Figure 2a). Post-hoc comparisons of the Interaction showed that control mice exposed to defeat

(Control+IRSD) performed less entries into the open arms than those in the Control+EXPL ($p<0.01$) and IMM+IRSD ($p<0.001$) groups.

The ANOVA of the latency to enter the open arms of the EPM revealed that the variable Inoculation [$F(1,39)=8.797$; $p<0.01$], Defeat [$F(1,39)=8.108$; $p<0.01$], and the Interaction of the variables Inoculation X Defeat [$F(1,39)=9.217$; $p<0.01$] were significant (Figure 2b). Post-hoc comparison of the Interaction showed that the Control+IRSD group displayed a higher latency to enter the open arms than the Control+EXPL ($p<0.01$) and IMM+IRSD ($p<0.001$) groups.

The ANOVA of the time spent in the open arms of the EPM revealed that the variable Inoculation [$F(1,39)=9.642$; $p<0.01$] and the Interaction of the variables Inoculation X Defeat [$F(1,39)=7.773$; $p<0.01$] were significant (Figure 2c). Post-hoc comparisons of the Interaction showed that control mice exposed to defeat (Control+IRSD) spent less time in the open arms of the EPM than mice in the Control+EXPL ($p<0.01$) and IMM+IRSD ($p<0.001$) groups.

The ANOVA of the percentage of entries into the open arms of the EPM revealed that the variable Defeat [$F(1,39)=6.906$; $p<0.05$] and the Interaction of the variables Inoculation X Defeat [$F(1,39)=9.250$; $p<0.01$] were significant (Figure 3a). Post-hoc comparison of the interaction highlighted a lower percentage of entries into the open arms by mice in the Control+IRSD group than by mice in the Control+EXPL ($p<0.001$) or IMM+IRSD ($p<0.01$) groups.

The ANOVA of the percentage of time spent in the open arms of the EPM revealed that only the variable Defeat was significant [$F(1,39)=5.365$;

$p<0.05$] (Figure 3b). Post-hoc comparison highlighted a lower percentage of entries into the open arms by mice in the defeated groups (Control+IRSD and IMM+IRSD) than in the non-defeated (Control+EXPL and IMM+EXPL) groups ($p<0.05$).

The ANOVA of the frequency of grooming revealed that only the variable Inoculation was significant [$F(1,39)=5.554$; $p<0.05$] (Figure 4a). Post-hoc comparison showed a lower frequency of grooming in mice exposed to immobilization (IMM+EXPL and IMM+IRSD groups) than in control mice (Control+EXPL and Control+IRSD groups) ($p<0.05$). The ANOVA of the time spent in grooming revealed that only the variable Defeat was significant [$F(1,39)=4.146$; $p<0.05$] (Figure 4b). Post-hoc comparison highlighted that mice in the defeated groups (Control+IRSD and IMM+IRSD) spent less time in grooming than those in the non-defeated groups (Control+EXPL and IMM+EXPL) ($p<0.05$).

The ANOVA of the ISI data revealed that the variable Inoculation [$F(1,39)=34.982$; $p<0.001$] and Defeat [$F(1,39)=18.516$; $p<0.001$] were significant (Figure 5). Post-hoc comparison of the variable Inoculation highlighted that mice exposed to immobilization (IMM+EXPL and IMM+IRSD groups) have a higher ISI than control mice (Control+EXPL and Control+IRSD groups) ($p<0.001$). In addition, post-hoc comparison of the variable Defeat showed that mice in the defeated groups (Control+IRSD and IMM+IRSD) have a lower ISI than those in the non-defeated groups (Control+EXPL and IMM+EXPL) ($p<0.001$).

The ANOVA of the number of dips revealed Inoculation to be the only significant variable [$F(1,39)=14.618$; $p<0.001$] (Figure 6). Mice exposed to immobilization (IMM+EXPL and IMM+IRSD groups) showed a higher

number of head dips than control mice (Control+EXPL and Control+IRSD groups) ($p<0.001$).

The ANOVA of the time spent in immobile in the TST revealed that that the variable Inoculation [$F(1,39)=4.039$; $p<0.05$] and Defeat [$F(1,39)=4.208$; $p<0.05$] were significant (Figure 7). Post-hoc comparison of the variable Inoculation highlighted that mice exposed to immobilization (IMM+EXPL and IMM+IRSD groups) spent less time immobile than control mice (Control+EXPL and Control+IRSD groups) ($p<0.05$). In addition, post-hoc comparison of the variable Defeat showed that mice in the defeated groups (Control+IRSD and IMM+IRSD) spent less time immobile than those in the non-defeated groups (Control+EXPL and IMM+EXPL) ($p<0.05$).

The ANOVA of the time spent in the drug-paired compartment revealed that the variable Days [$F(1,37)=22.611$; $p<0.05$], the interaction Inoculation X Defeat [$F(1,37)=4.605$; $p<0.05$], and the interaction Days X Inoculation X Defeat [$F(1,37)=4.316$; $p<0.05$] were significant (Figure 8). Post-hoc comparison of the interaction Days X Inoculation X Defeat showed that mice in the Control+IRSD and IMM+EXPL groups spent more time in the drug-paired compartment in Post-C than in Pre-C ($p<0.001$ and $p<0.05$, respectively). In addition, on Post-C day the group Control+IRSD spent more time in drug-paired compartment than the Control+EXPL and IMM+IRSD groups ($ps<0.05$).

Exposure to an acute Social Defeat episode on early adolescence

The ANOVA of the number of entries into the open arms of the EPM revealed that the variables Inoculation [$F(1,40)=7.291$; $p<0.01$] and Defeat [$F(1,40)=6.466$; $p<0.05$] were significant (Figure 2a). Post-hoc comparison

of the variable Inoculation highlighted that mice exposed to an acute defeat (SD+EXPL and SD+IRSD groups) performed more entries into the open arms than control mice (Control+EXPL and Control+IRSD groups) ($p<0.01$). In addition, post-hoc comparison of the variable Defeat showed that mice in the defeated groups (Control+IRSD and SD+IRSD) performed less entries into the open arms than those in the non-defeated groups (Control+EXPL and SD+EXPL) ($p<0.05$).

The ANOVA of the latency to enter the open arms of the EPM revealed that the variable Inoculation [$F(1,40)=6.411$; $p<0.05$], Defeat [$F(1,40)=8.333$; $p<0.01$], and the Interaction of the variables Inoculation X Defeat [$F(1,40)=5.844$; $p<0.05$] were significant (Figure 2b). Post-hoc comparison of the Interaction showed that the Control+IRSD group displayed a higher latency to enter the open arms than the Control+EXPL ($p<0.01$) and IMM+IRSD ($p<0.001$) groups.

The ANOVA of the time spent in the open arms of the EPM revealed that only the variable Defeat was significant [$F(1,40)=9.955$; $p<0.01$] (Figure 2c). Post-hoc comparisons showed that mice exposed to repeated defeat (Control+IRSD and SD+IRSD) spent less time in the open arms of the EPM than mice non defeated in late adolescence (Control+EXPL and SD+EXPL groups) ($p<0.01$).

The ANOVA of the percentage of entries into the open arms of the EPM revealed that the variable Defeat [$F(1,40)=6.862$; $p<0.05$] and the Interaction of the variables Inoculation X Defeat [$F(1,40)=3.94$; $p<0.05$] were significant (Figure 3a). Post-hoc comparison of the interaction highlighted a lower percentage of entries into the open arms by mice in the Control+IRSD group than by mice in the Control+EXPL ($p<0.01$) group.

The ANOVA of the percentage of time spent in the open arms of the EPM revealed that only the variable Defeat was significant [$F(1,40)=10.201$; $p<0.01$] (Figure 3b). Post-hoc comparison highlighted a lower percentage of entries into the open arms by mice in the defeated groups (Control+IRSD and SD+IRSD) than in the non-defeated (Control+EXPL and SD+EXPL) groups ($p<0.01$).

The ANOVA of the frequency of grooming revealed that only the variable Inoculation was significant [$F(1,40)=7.811$; $p<0.05$] (Figure 4a). Post-hoc comparison showed a lower frequency of grooming in mice exposed to an acute social defeat (SD+EXPL and SD+IRSD groups) than in control mice (Control+EXPL and Control+IRSD groups) ($p<0.05$). The ANOVA of the time spent in grooming did not reveal significant effects (Figure 4b).

The ANOVA of the ISI data revealed that the variable Inoculation [$F(1,40)=16.351$; $p<0.001$], Defeat [$F(1,40)=11.849$; $p<0.001$], and the interaction Inoculation X Defeat [$F(1,40)=4.05$; $p<0.05$] were significant (Figure 5). Post-hoc comparison of the interaction highlighted a lower ISI in the Control+IRSD group than in the Control+EXPL ($p<0.001$) or SD+IRSD ($p<0.001$) groups.

The ANOVA of the number of dips did not reveal significant effects (Figure 6).

The ANOVA of the time spent in immobile in the TST revealed that that the variable Defeat [$F(1,39)=5.124$; $p<0.05$] and the interaction Inoculation X Defeat [$F(1,39)=6.141$; $p<0.05$] were significant (Figure 7). Post-hoc comparison of the interaction showed that mice of the group Control+IRSD spent less time immobile than mice of the group Control+EXPL ($p<0.01$).

The ANOVA of the time spent in the drug-paired compartment revealed that the variable Days [$F(1,37)=12.972$; $p<0.001$], the interaction Days X Inoculation [$F(1,37)=5.815$; $p<0.05$], and the interaction Days X Inoculation X Defeat [$F(1,37)=4.056$; $p<0.05$] were significant (Figure 8). Post-hoc comparison of the interaction Days X Inoculation X Defeat showed that mice in the Control+IRSD group spent more time in the drug-paired compartment in Post-C than in Pre-C ($p<0.001$). In addition, on Post-C day the group Control+IRSD spent more time in drug-paired compartment than the Control+EXPL and SD+IRSD groups ($ps<0.01$).

Exposure to Vicarious Social Defeat on early adolescence

The ANOVA of the number of entries into the open arms of the EPM revealed that the variables Inoculation [$F(1,39)=14.260$; $p<0.001$] and Defeat [$F(1,39)=13.102$; $p<0.001$] were significant (Figure 2a). Post-hoc comparison of the variable Inoculation highlighted that mice exposed to vicarious social defeat (VSD+EXPL and VSD+IRSD groups) performed more entries into the open arms than control mice (Control+EXPL and Control+IRSD groups) ($p<0.01$). In addition, post-hoc comparison of the variable Defeat showed that mice in the defeated groups (Control+IRSD and VSD+IRSD) performed less entries into the open arms than those in the non-defeated groups (Control+EXPL and VSD+EXPL) ($p<0.01$).

The ANOVA of the latency to enter the open arms of the EPM revealed that the variable Inoculation [$F(1,39)=14.634$; $p<0.001$], Defeat [$F(1,39)=8.621$; $p<0.01$], and the Interaction of the variables Inoculation X Defeat [$F(1,39)=9.977$; $p<0.01$] were significant (Figure 2b). Post-hoc comparison of the Interaction showed that the Control+IRSD group displayed a higher

latency to enter the open arms than the Control+EXPL and IMM+IRSD groups (ps<0.001).

The ANOVA of the time spent in the open arms of the EPM revealed that the variables Inoculation [$F(1,39)=4.965$; $p<0.05$] and Defeat [$F(1,39)=11.782$; $p<0.001$] were significant (Figure 2c). Post-hoc comparisons of the variable Inoculation highlighted that mice exposed to vicarious social defeat (VSD+EXPL and VSD+IRSD groups) spent more time in the open arms than control mice (Control+EXPL and Control+IRSD groups) ($p<0.05$). Post-hoc comparison of the variable Defeat showed that mice exposed to repeated defeat (Control+IRSD and VSD+IRSD) spent less time in the open arms of the EPM than mice non defeated in late adolescence (Control+EXPL and VSD+EXPL groups) ($p<0.001$).

The ANOVA of the percentage of entries into the open arms of the EPM revealed that only the variable Defeat was significant [$F(1,39)=13.805$; $p<0.001$] (Figure 3a). Post-hoc comparison highlighted a lower percentage of entries into the open arms by mice in the defeated groups (Control+IRSD and VSD+IRSD) than in the non-defeated (Control+EXPL and VSD+EXPL) groups ($p<0.001$).

The ANOVA of the percentage of time spent in the open arms of the EPM revealed that only the variable Defeat was significant [$F(1,39)=6.769$; $p<0.05$] (Figure 3b). Post-hoc comparison highlighted a lower percentage of time in the open arms by mice in the defeated groups (Control+IRSD and VSD+IRSD) than in the non-defeated (Control+EXPL and VSD+EXPL) groups ($p<0.05$).

The ANOVA of the frequency of grooming did not reveal significant effects (Figure 4a). The ANOVA of the time spent in grooming revealed that only the variable Defeat was significant [$F(1,39)=4.281$; $p<0.05$] (Figure 4b). Post-hoc comparison highlighted that mice in the defeated groups (Control+IRSD and VSD+IRSD) spent less time in grooming than those in the non-defeated groups (Control+EXPL and VSD+EXPL) ($p<0.05$).

The ANOVA of the ISI data revealed that only the variable Defeat was significant [$F(1,39)=5.070$; $p<0.05$] (Figure 5). Post-hoc comparison of the variable Defeat showed that mice in the defeated groups (Control+IRSD and VSD+IRSD) have a lower ISI than those in the non-defeated groups (Control+EXPL and VSD+EXPL) ($p<0.05$).

The ANOVA of the number of dips revealed Defeat to be the only significant variable [$F(1,39)=5.04$; $p<0.05$] (Figure 6). Mice exposed to repeated defeat (Control+IRSD and VSD+IRSD groups) showed a lower number of head dips than mice not exposed to repeated defeat (Control+EXPL and VSD+EXPL groups) ($p<0.05$).

The ANOVA of the time spent in immobile in the TST revealed that that the variable Defeat [$F(1,39)=4.291$; $p<0.05$] and the interaction Inoculation X Defeat [$F(1,39)=5.524$; $p<0.05$] were significant (Figure 7). Post-hoc comparison of the interaction showed that mice of the groups Control+IRSD ($p<0.01$) and VSD+EXPL ($p<0.05$) spent less time immobile than mice of the group Control+EXPL.

The ANOVA of the time spent in the drug-paired compartment revealed that only the variable Days [$F(1,37)=8.291$; $p<0.05$] was significant (Figure 8).

Simple Effects of this variable showed that the effect of Days was only significant in the Control+IRSD group [$F(1,37)=9.055$; $p<0.01$].

CONCLUSIONS

Exposure to three different protocols of inoculation to stress in early adolescence (acute immobilization, acute social defeat and acute vicarious social defeat) prevent some of the short-term effects of IRSD exposure in late adolescence (depending on the protocol of inoculation employed) and promotes resilience to the long-term effects of IRSD on cocaine reward. Mice exposed to IRSD in late adolescence develop cocaine CPP, but defeated mice previously exposed to these stressful events do not develop CPP. These results support the hypothesis of stress inoculation.

- **Effects of acute immobilization**

Exposure to an acute episode of immobilization in early adolescence induces by itself (without IRSD) behavioral effects in late adolescent and adult mice. Thirty days after exposure to immobilization, mice without additional stress exposure (IMM+EXPL) show an increase in novelty-seeking behavior and social interaction but a reduction of grooming and immobility in the TST. Furthermore, mice exposed only to immobilization in early adolescence acquire cocaine CPP in adulthood.

Acute immobilization in early adolescence prevents some of the short-term effects of IRSD exposure in late adolescence, such as anxiety-like behavior,

the reduction of novelty-seeking and social avoidance. However, other effects of social stress (decrease in grooming behavior and immobility in the TST) remain unaltered in IMM+IRSD mice.

In addition, acute immobilization in early adolescence promotes resilience to the long-term effects of IRSD on cocaine reward. Mice exposed to IRSD in late adolescence develop cocaine CPP, but defeated mice previously exposed to immobilization do not develop CPP.

- **Effects of acute social defeat**

Exposure to ASD in early adolescence induces by itself (without additional stress exposure) a reduction of grooming behavior thirty days after the episode of ASD.

Exposure to ASD in early adolescence prevents some short-term effects of IRSD exposure in late adolescence, such as the reduction of novelty-seeking, social avoidance and immobility in the TST. Other effects of social stress (anxiety-like behavior and a decrease in grooming behavior) remain unaltered by ASD.

In addition, ASD in early adolescence promotes resilience to the long-term effects of IRSD on cocaine reward. Mice exposed to IRSD in late adolescence develop cocaine CPP, but defeated mice previously exposed to a social defeat do not develop CPP.

- **Effects of vicarious social defeat**

Exposure to acute VSD in early adolescence induces by itself (without additional stress exposure) a reduction of grooming behavior and immobility in the TST thirty days after the episode of VSD.

Exposure to an acute episode of VSD in early adolescence only prevents the decrease in immobility in the TST induced by IRSD exposure in late adolescence, while the other short-term effects of IRSD (anxiety-like behavior, reduction of novelty-seeking, social avoidance and decrease in grooming behavior) remain unaltered.

In addition, VSD in early adolescence promotes resilience to the long-term effects of IRSD on cocaine reward. Mice exposed to IRSD in late adolescence develop cocaine CPP, but defeated mice previously exposed to VSD do not develop CPP.

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SUPPLEMENTARY MATERIAL

Fig. 1a. Protocols of inoculation

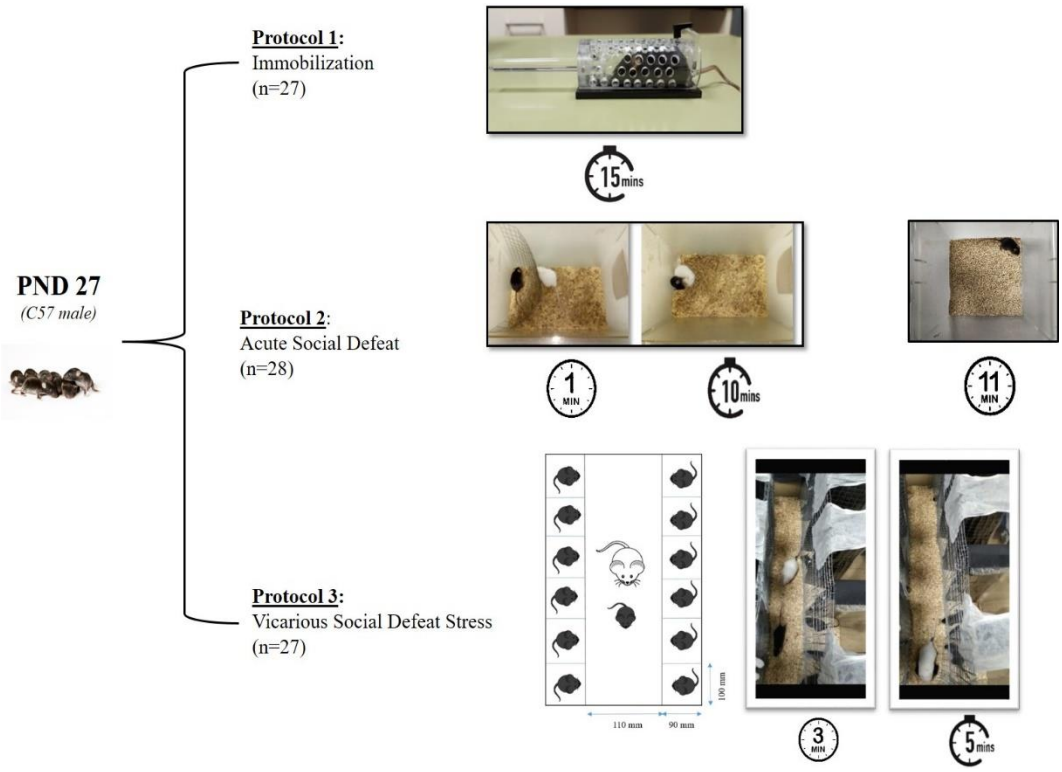


Fig. 1b. Experimental design

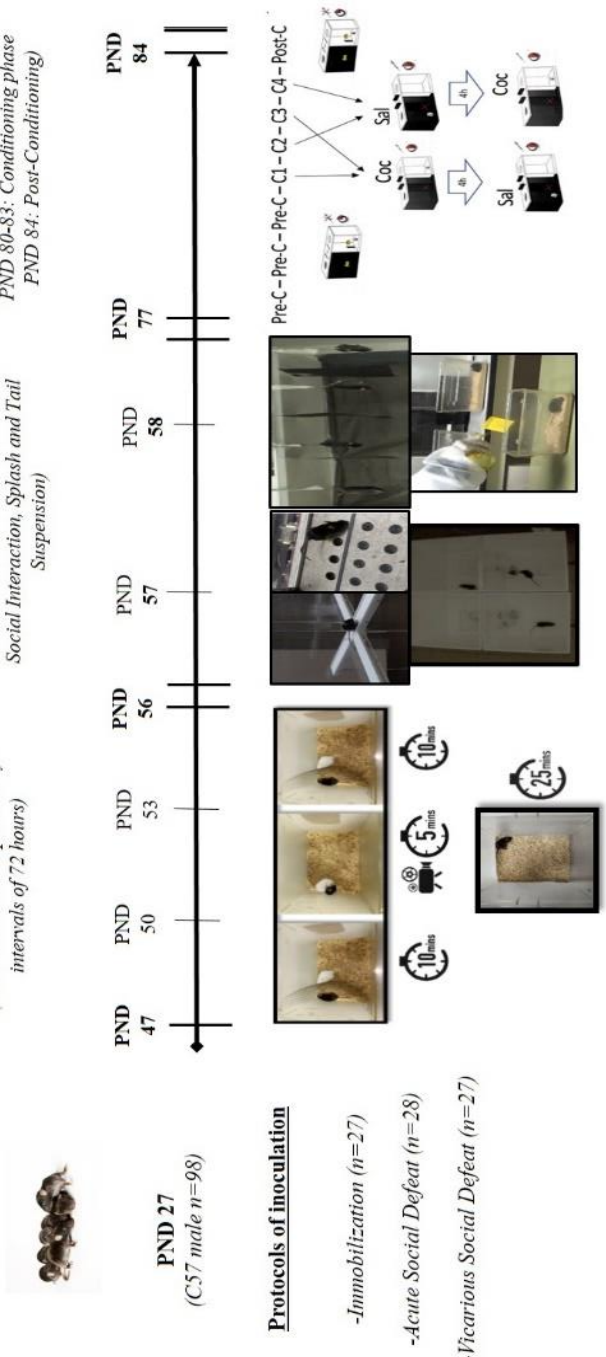


Fig. 2a

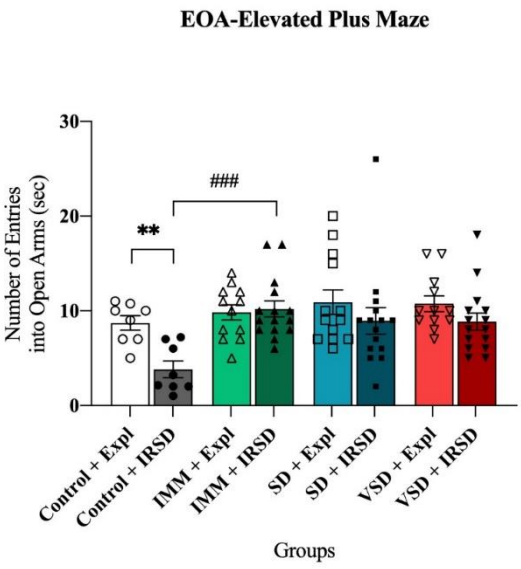


Fig. 2b

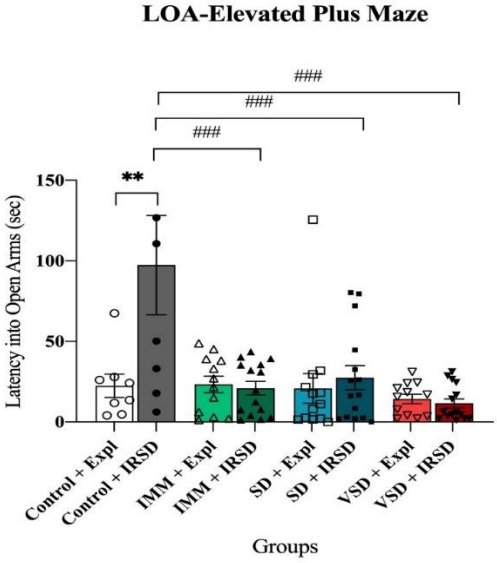


Fig. 2c

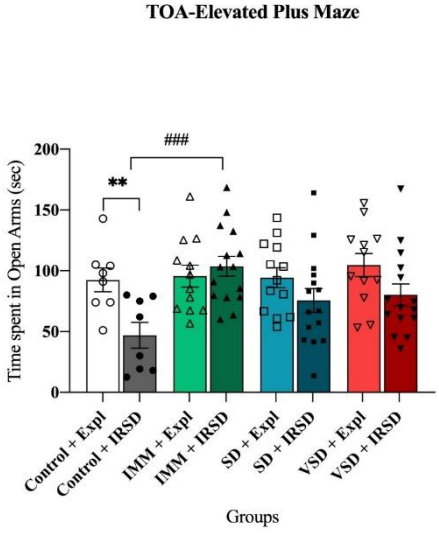


Fig. 3a

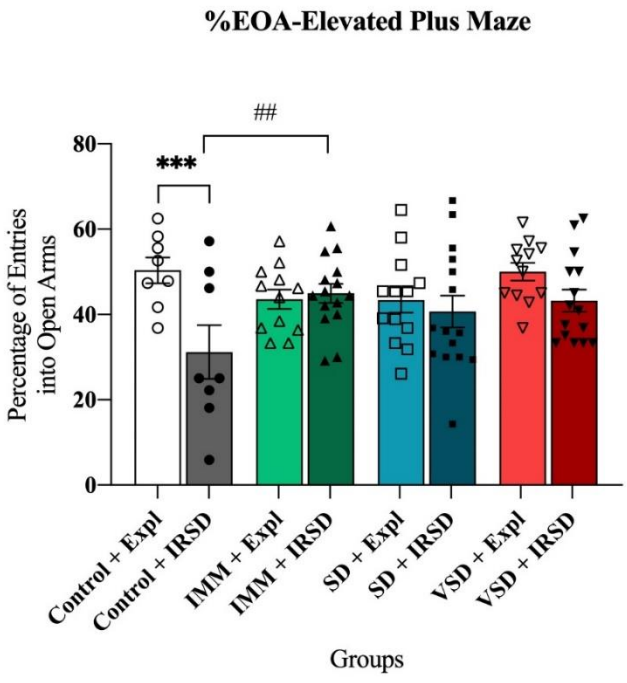

%EOA-Elevated Plus Maze
##
***
Percentage of Entries into Open Arms
80
60
40
20
0
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 3b

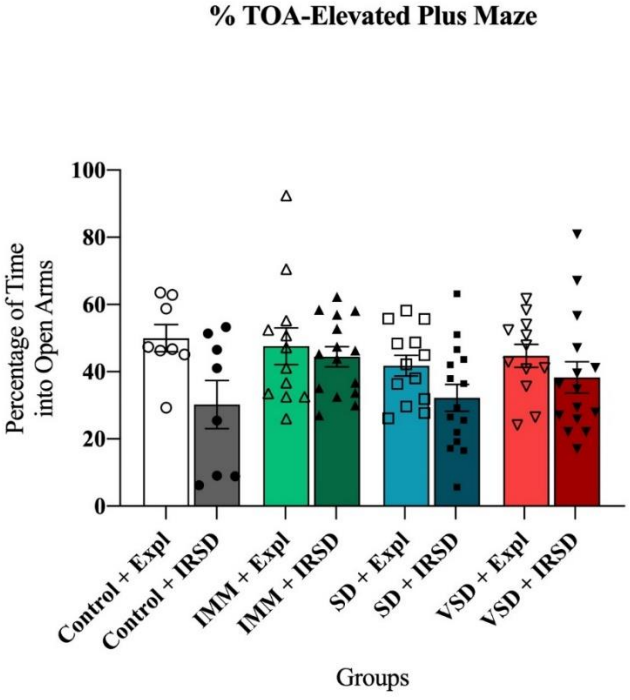

% TOA-Elevated Plus Maze
Percentage of Time into Open Arms
100
80
60
40
20
0
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 4a

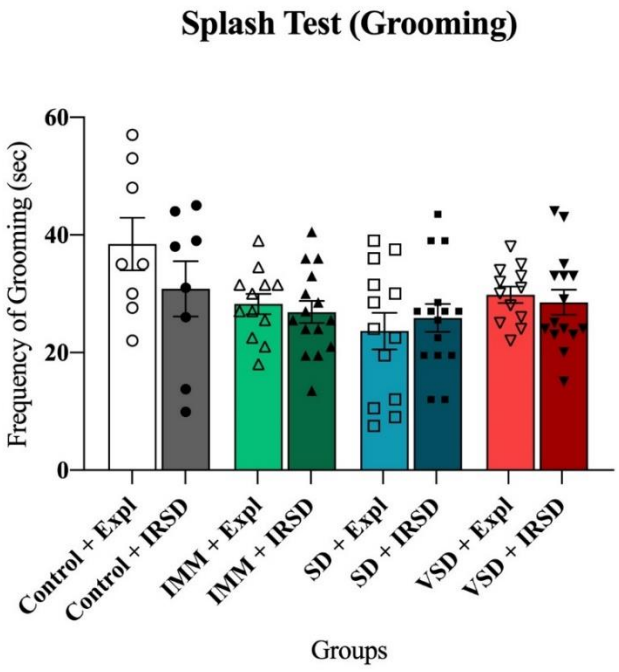

Splash Test (Grooming)
Frequency of Grooming (sec)
60
40
20
0
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 4b

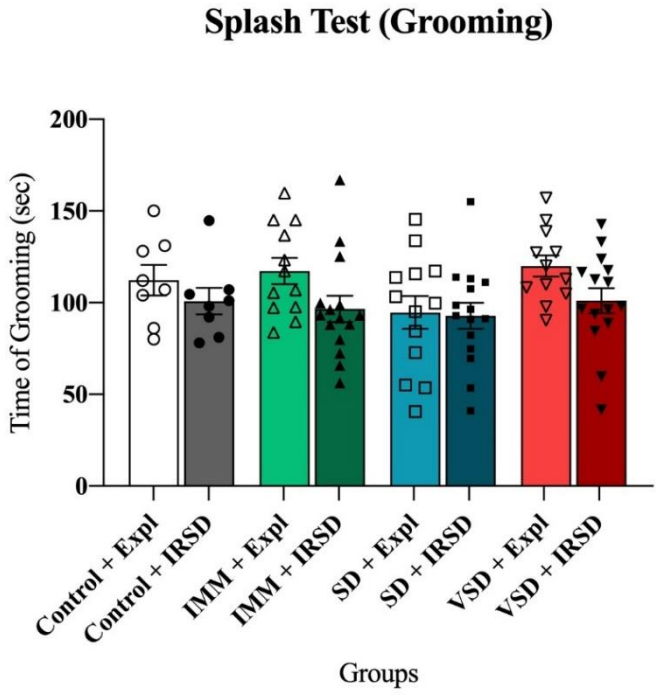

Splash Test (Grooming)
Time of Grooming (sec)
200
150
100
50
0
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 5

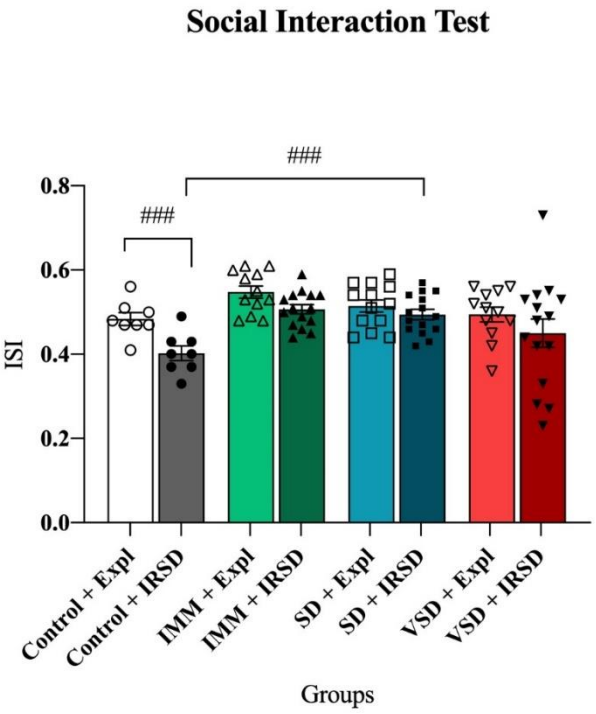

Social Interaction Test
###
###
0.8
0.6
0.4
0.2
0.0
ISI
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 6

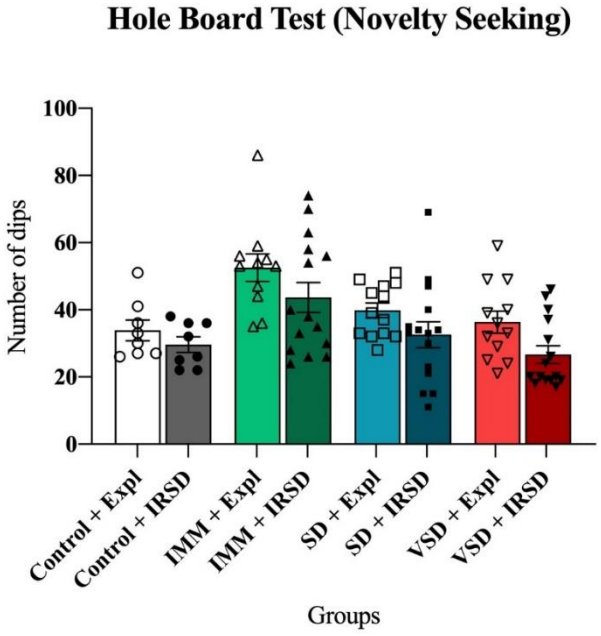

Hole Board Test (Novelty Seeking)
100
80
60
40
20
0
Number of dips
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig. 7

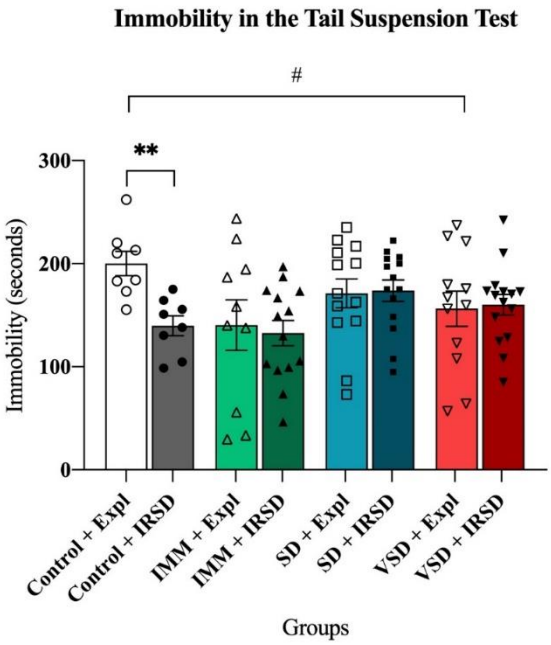
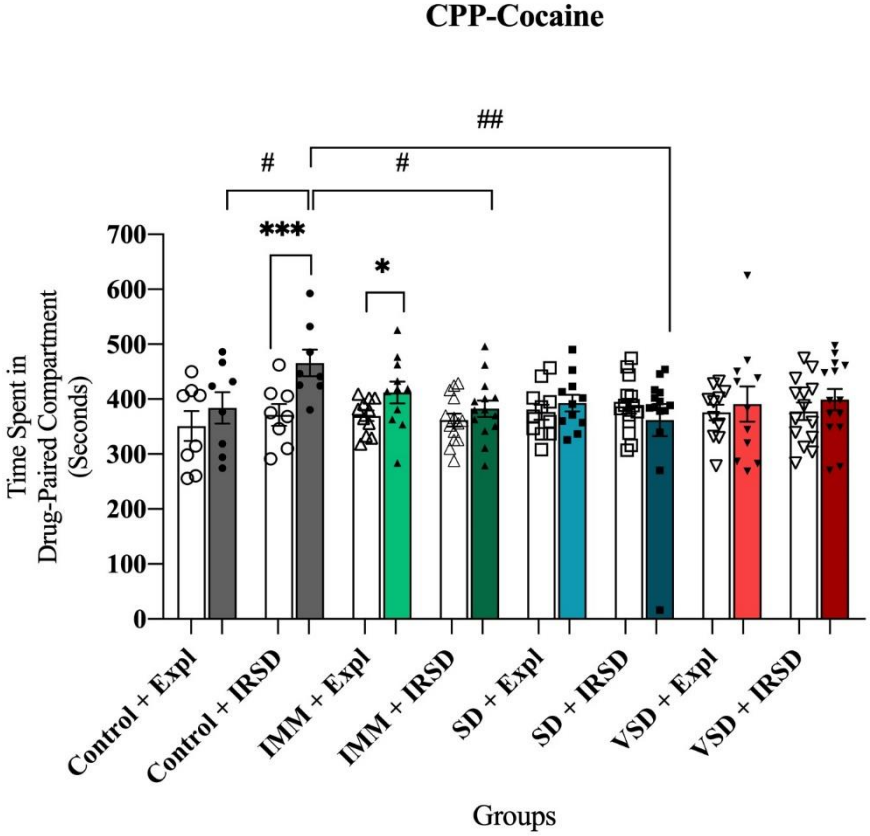

Immobility in the Tail Suspension Test
#
**
300
200
100
0
Immobility (seconds)
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Fig 8.


CPP-Cocaine
##
#
#
***
*
700
600
500
400
300
200
100
0
Time Spent in Drug-Paired Compartment (Seconds)
Control + Expl
Control + IRSD
IMM + Expl
IMM + IRSD
SD + Expl
SD + IRSD
VSD + Expl
VSD + IRSD
Groups

Experimental Study 5

(Under review)

Drug and Alcohol Dependence

Resilience to the Short- and Long-term Behavioral Effects of Intermittent Repeated Social Defeat in Adolescent Male Mice.

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Section/Category:	Behavioral pharmacology - Animal
Keywords:	resilience; social defeat stress; cocaine; adolescence; male mice; conditioned place preference
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	MARIA Asuncion AGUILAR, Ph.D.
Abstract:	Background. Exposure to intermittent repeated social defeat (IRSD) increases the sensitivity of mice to the rewarding effects of cocaine in the conditioned place preference (CPP) paradigm. Some animals are resilient to this effect of IRSD, though research exploring this inconsistency in adolescent mice is scarce. Thus, our aim was to characterize the behavioral profile of mice exposed to IRSD during early adolescence and to explore a potential association with resilience to the short- and long-term effects of IRSD. Methods. Thirty-six male C57BL/6 mice were exposed to IRSD during early adolescence (PND 27, 30, 33 and 36), while another 10 male mice did not undergo stress (controls). Defeated mice and controls then carried out the following battery of behavioral tests; the Elevated Plus Maze, Hole-Board and Social Interaction Test on PND 37, and the Tail Suspension and Splash tests on PND 38. Three weeks later, all the mice were submitted to the CPP paradigm with a low dose of cocaine (1.5 mg/kg). Results. IRSD during early adolescence induced depressive-like behavior in the Social Interaction and Splash tests and increased the rewarding effects of cocaine. Mice with low levels of submissive behavior during episodes of defeat were resilient to the short- and long-term effects of IRSD. In addition, resilience to the short-term effects of IRSD on social interaction and grooming behavior predicted resilience to the long-term effects of IRSD on cocaine reward. Conclusion. Our findings help to characterize the nature of resilience to the effects of social stress during adolescence.
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Contributors: MAA and MPG-P contributed to the conception and design of the study. CC-L and MAM-C, performed the experiments, organized the databases and performed the statistical analyses. CC-L and MPG-P wrote the first draft of the manuscript. MAA wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of Interest: No conflict declared.

Acknowledgments: We acknowledge the assistance of Brian Normanly with the English language editing.

Highlights (for review)

Some mice are resilient to the effects of social defeats during early adolescence

Low submission during defeats predicts resilience to depression-like behavior

Low submission during defeats predicts resilience to potentiation of cocaine reward

Resilience to depression-like behavior predicts resilience to acquire cocaine reward

Resilience to the Short- and Long-term Behavioral Effects of Intermittent Repeated Social Defeat in Adolescent Male Mice.

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ABSTRACT

Background. Exposure to intermittent repeated social defeat (IRSD) increases the sensitivity of mice to the rewarding effects of cocaine in the conditioned place preference (CPP) paradigm. Some animals are resilient to this effect of IRSD, though research exploring this inconsistency in adolescent mice is scarce. Thus, our aim was to characterize the behavioral profile of mice exposed to IRSD during early adolescence and to explore a potential association with resilience to the short- and long-term effects of IRSD. Methods. Thirty-six male C57BL/6 mice were exposed to IRSD during early adolescence (PND 27, 30, 33 and 36), while another 10 male mice did not undergo stress (controls). Defeated mice and controls then carried out the following battery of behavioral tests; the Elevated Plus Maze, Hole-Board and Social Interaction Test on PND 37, and the Tail Suspension and Splash tests on PND 38. Three weeks later, all the mice were submitted to the CPP paradigm with a low dose of cocaine (1.5 mg/kg). Results. IRSD during early adolescence induced depressive-like behavior in the Social Interaction and Splash tests and increased the rewarding effects of cocaine. Mice with low levels of submissive behavior during episodes of defeat were resilient to the short- and long-term effects of IRSD. In addition, resilience to the short-term effects of IRSD on social interaction and grooming behavior predicted resilience to the long-term effects of IRSD on cocaine reward. Conclusion. Our findings help to characterize the nature of resilience to the effects of social stress during adolescence.

Keywords: resilience, social defeat stress, cocaine, adolescence, mice, conditioned place preference

Abbreviations: *CPP, conditioned place preference; EPM, elevated plus maze; FG, frequency of grooming; ISI, index of social interaction; NS, novelty-seeking; PND, post-natal day; IRSD, intermittent repeated social defeat; TG, time in grooming; TI, time of immobility; TST, tail suspension test; %TOA, percentage of time in the open arms.*

1. INTRODUCTION

Adolescence is a critical developmental stage characterized by physical, emotional, cognitive and behavioral changes during which the process of brain maturation is completed (Spear, 2000). For this reason, the adolescent brain is particularly sensitive to stressors (Romeo, 2017). Studies in animal models have demonstrated that exposure to stress during adolescence alters brain development, increasing vulnerability to the development of anxiety, depression and drug use disorders in adulthood (al´Absi, 2020; Burke et al., 2017). While chronic stress is generally associated with the appearance of psychopathologies, recent studies have shown that, under certain circumstances, exposure to stress in early life or adolescence can promote resilience to future stressful events (Calpe-López et al., 2022a; Cotella et al., 2022; Mancini et al., 2021; Ordoñes Sanchez et al., 2021).

In previous works, we have studied the long-term consequences of social stress on the vulnerability of mice to drugs of abuse by exposing animals to a protocol of intermittent repeated social defeat (IRSD) by an aggressive adult conspecific animal. This paradigm has been demonstrated to be useful for modelling social stress, which is the most frequent type of stress faced by human beings (Calpe-López 2022a, 2022b, 2022c; García-Pardo et al., 2015, 2022). Mice are usually exposed to defeat during late adolescence or adulthood; however, the use of early adolescent mice may be of greater relevance, as it models the human context in which bullying takes place. We have previously demonstrated that exposure to IRSD in early or late adolescence enhances the vulnerability of mice to the rewarding effects of cocaine (Calpe-López et al., 2020; Montagud-Romero et al., 2017; Rodríguez-Arias et al., 2017) and MDMA (García-Pardo et al., 2015) in the conditioned place preference (CPP) paradigm.

As explained previously, an increasing number of studies are focusing on resilience to the effects of stress rather than vulnerability (Calpe-López et al., 2022a). As in humans, not all mice exposed to social stress develop depression-, anxiety- or addictive-like disorders (Krishnan et al., 2007; Russo et al., 2012; Schmidt et al., 2010). In a previous study we observed an increase in the rewarding effects of cocaine in adulthood in a subset of mice exposed to IRSD during late adolescence (susceptible mice), while another subset showed resilience to the negative effects of stress on cocaine reward (Calpe-López et al., 2020). In the same study we demonstrated that vulnerability and resilience to the effects of IRSD on late adolescence were associated with different behavioral profiles. Resilient mice were characterized by less submission during defeat episodes, less interest in the open arms in the elevated plus maze

(EPM), less novelty-seeking in the hole-board test, less reactivity in the tail suspension test (TST), and an absence of RSD-induced deficits such as social avoidance in the social interaction test and anhedonia in the splash test (Calpe-López et al., 2020).

Resilience to the negative consequences of social defeat stress in early adolescence has been less studied. It has been suggested that the responses of adolescent mice exposed to different paradigms of social defeat are more complex and singular in comparison to those of mice defeated in adulthood. Adult mice exposed to chronic social defeat stress (CSDS) can be characterized according to their levels of social interaction and sucrose preference as susceptible or resilient to CSDS (Krishnan et al., 2007; Russo et al., 2012). Conversely, it has been observed that only a small proportion of mice defeated in adolescence were either totally susceptible or totally resilient in both social interaction and sucrose preference tests, while most were susceptible in one test and resilient in the other (Alves-Dos-Santos et al., 2020). Similar age differences seem to exist in resilience to the effects of IRSD. Adult mice exposed to IRSD showed a consistent resilient phenotype to depressive-like behaviors (in the social interaction and splash tests) and to an increase in cocaine reward (Ballestín et al., 2021; Calpe-López et al., 2020) and alcohol consumption (Reguilón et al., 2021). However, mice exposed to IRSD during adolescence and which displayed resilience to depressive-like behaviors in the social interaction test exhibited more anxiety in the EPM, an increased preference for cocaine-paired compartment and greater ethanol consumption (Reguilón et al., 2022).

Thus, the aim of this work was to characterize the behavioral profile of mice exposed to IRSD during early adolescence and to evaluate the potential association of this profile with resilience to the short- and long-term effects of IRSD. We have followed exactly the same procedure as that used in a previous study with late adolescent mice (Calpe-López et al., 2020), the only difference being the post-natal days (PND) on which defeat was experienced. In this way, we could compare the phenotype of resilient mice exposed to IRSD in early (PND 27-36) versus late (PND 47-56) adolescence.

2. MATERIAL AND METHODS

2.1. Subjects

A total number of 46 male mice of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were delivered to our laboratory at 21 days of age and 42 days of age, respectively. Experimental mice (C57BL/6) were housed in groups (n=4-5) while mice

used as aggressive opponents (OF1) were housed individually to induce heightened aggression (Rodríguez-Arias et al., 1998). All mice were housed under standard laboratory conditions (see details in Calpe-López et al., 2020). All procedures were conducted according the Directive 2010/63/EU and were approved by the Ethics Committee of Experimental Research of the University of Valencia (A1507028485045).

2.2. Drugs

Animals were injected intraperitoneally with 1.5 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) in a volume of 0.01 ml/g of weight. Physiological saline was also used to dissolve the cocaine. The dose of cocaine was selected on the basis of a study evaluating resilience to the effects of IRSD in early adolescent mice on cocaine CPP (Reguilón et al., 2022).

2.3. Experimental Design

Following the adaptation period, the experimental mice (C57BL/6) were assigned to two groups: a non-stressed control group (n = 10) and a group subsequently exposed to four episodes of IRSD (n = 36) on PND 27, 30, 33 and 36. On PND 37–38, all the mice underwent different behavioral tests to evaluate the short-term effects of IRSD. On PND 37, all the mice performed the elevated plus maze (EPM), the hole board (HB) and the social interaction tests. On PND 38, all the mice performed the tail suspension test (TST) and the splash test. The animals were then housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (PND 57-64) (see Figure 1). All experiments took place during the dark period (8.30–16.30) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h

prior to testing. All the experimental protocols were performed as is described in Calpe-López et al. (2020). In addition, detailed information of the experimental protocols can be found in the Supplementary material.

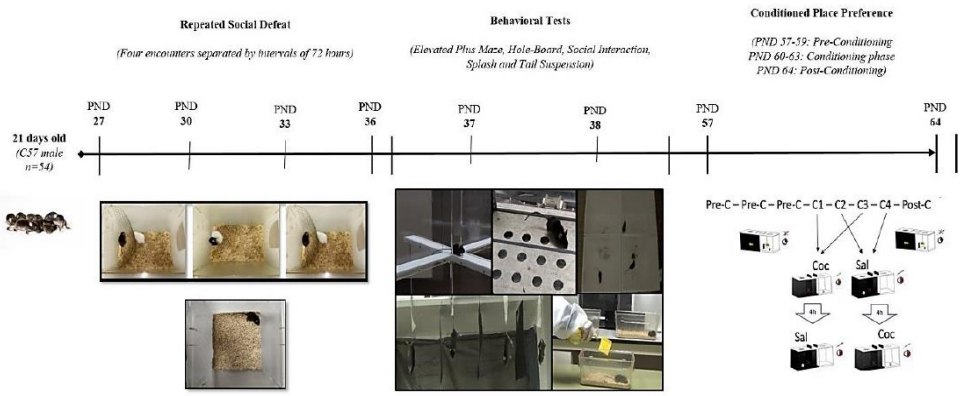

Figure 1. Experimental Design. Two groups of mice were used. One group was exposed to intermittent repeated social defeat (IRSD, n = 36). On postnatal day (PND) 27, 30, 33 and 36, experimental mice were introduced into the cage of an aggressive opponent. The physical contact between them was allowed for 5 min, during which the experimental mouse experienced social defeat (SD). On the same PND, the mice in the other group (CONTROL, n = 10) explored an empty cage. On PND 37, all mice performed the elevated plus maze (EPM), the hole board (HB) and social interaction (SI) tests. On PND 38, all mice performed the tail suspension test (TST) and the splash test. After an interval of 3 weeks, all mice underwent the conditioned place preference (CPP) paradigm. On PND 57, 58 and 59, they underwent the pre-conditioning (Pre-C) phase. On PND 60, 61, 62, 63 they performed four conditioning sessions (C1-C4), receiving 1.5 mg/kg of cocaine (Coc) or saline (Sal) before being placed in the drug- or saline-paired compartment, respectively. On PND 64, mice underwent the post-conditioning (Post-C) phase.

2.5. Statistical Analysis

The effects of RSD on the different behavioral measures (with the exception of CPP) were evaluated by means of unpaired Student t-tests (Control vs. IRSD). In the case of CPP, a mixed two-way ANOVA with a within-subjects variable – Days, with two levels (Pre-C and Post-C) - and a between-subjects variable – Stress, with two levels (Control and IRSD) - was used. Post hoc comparisons were performed with Simple Effects and Bonferroni tests.

With the data obtained in the defeat episodes and in the behavioral tests (EPM, hole board, social interaction, tail suspension and splash tests), the group of defeated mice was separated into two subgroups (High or Low Score group) according to the median of the whole group (see detailed information in Calpe-López et al. (2020) and Supplementary Material). Unpaired Student´s t tests were performed between mice showing High and Low submissive behavior (time in defense/submission in the first episode of defeat), for the behavior shown during the first and fourth episodes of defeat, and for the measurements of percentage of time in the open arms of the EPM, number of dips in the hole board, time immobile in the TST, and grooming (frequency and time) in the splash test. To determine the influence of the different behavioral profiles on resilience to the short-term behavioral effects of IRSD, a one-way ANOVA with a between-subjects variable—Group, with three levels (Control, IRSD High Score and IRSD Low Score)—was performed for the percentage of time spent in the open arms of the EPM, number of dips in the hole board, time immobile in the TST, and grooming (frequency and time) in the splash test. The post hoc comparisons were performed with Bonferroni tests.

To determine the possible behavioral markers of resilience to the effects of social defeat on cocaine CPP, a mixed two-way ANOVA with a within-subjects variable—Days, with two levels (Pre-C and Post-C)—and a between-subjects variable—Group, with three levels (Control, IRSD High Score and IRSD Low Score)—was used for all the measures described above. Post hoc comparisons were performed with Bonferroni tests. In order to determine whether there was a relationship between the performance of mice in the short-term behavioral tests and in the CPP, Pearson correlation tests were used (see Supplementary material). For this purpose, the conditioning score (time spent in Post-C minus time spent in Pre-C) was calculated. All statistical analyses were performed with the SPSS program.

3. RESULTS

3.1. Behavioral effects of IRSD

Regarding the short-term behavioral effects of IRSD, the Student t test comparing Control and IRSD-exposed mice revealed that the experience of IRSD during early adolescence only induced a significant deficit in the ISI [$t(44)=5.342$; $p<0.001$] (see Figure 2a) and a reduction in frequency of grooming [$t(44)=1.711$; $p<0.05$] (see Figure 2b). No significant

differences were observed in the measurements obtained in the EPM, hole-board and tail suspension procedures.

Regarding the long-term effects of IRSD on cocaine reward, the ANOVA of the CPP data showed that the variable Days was significant [F (1,40) = 8.549; p<0.01], while the variables Stress and the interaction Days x Stress were not. However, Simple Effects of Days revealed that the increase in the time spent in the drug-paired compartment between Pre- and Post-C days was significant only in the group of mice exposed to IRSD [F (1,40) = 18.375; p<0.001] (see Figure 2c).

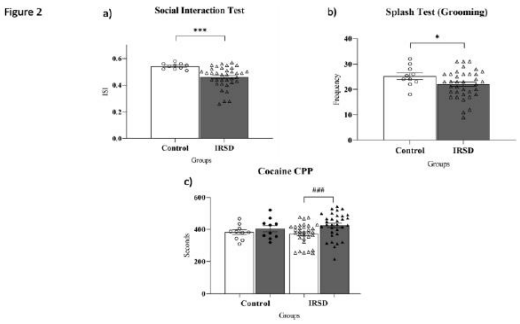

Figure 2. Behavioral effects of ISRD. One group of early adolescent male mice was not exposed to stress (CONTROL, n = 10) and the other group was exposed to IRSD on PND 27, 30, 33 and 36 (n = 36). **a)** Short-term effects of IRSD on the social interaction test. Bars represent the mean (±SEM) ISI. ***P < 0.001, significant difference with respect to the CONTROL group. **b)** Short-term effects of IRSD on the frequency of grooming behavior in the splash test. *P < 0.05, significant difference with respect to the CONTROL group. **c)** Long-term effects of IRSD on cocaine-induced CPP. All mice (CONTROL and IRSD groups) were conditioned with 1.5 mg/kg of cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, grey bars). ###P < 0.001, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.2. Behavioral Profile of Mice During Social Defeat and Resilience to the effects of IRSD

According to their scores in the time spent engaged in Defense/Submission behavior during the first episode of defeat, defeated mice were separated into two subgroups as mice with Low or High Submissive behavior (below or above the median of the defeated group, which was 55 sec; see Table 1). Student t test showed that these groups differed significantly regarding the time spent in defense/submission [t (25) = -6.374; p<0.001], and the frequency

[t (34) = 2.564; p<0.01] and latency [t (34) = 2.564; p<0.01] of this behavior in the first episode of defeat. In addition, Low Submissive mice spent less time [t (34) = -2.581; p<0.01] and showed a lower frequency [t (34) = -3.036; p<0.01] of submission in the fourth episode of defeat. Low submissive mice also received a lower number of threats from opponents in the first [t (34) = -2.267; p<0.05] and fourth [t (34) = -2.351; p<0.05] episodes of defeat.

			1st SD		4th SD	
			IRSD Low Submission	IRSD High Submission	IRSD Low Submission	IRSD High Submission
Experimental (intruder) mice	Defense/Submission	Frecuency	7,55 (+ 1)	14,68 (+ 0,95) +++	8,61 (+ 0,95)	12,44 (+ 1,71) ++
		Latency	28,47 (± 6,8)	9,79 (± 2,57) ++	19,02 (± 4,41)	24,93 (± 8,66)
		Time	29,61 (± 3,73)	79,72 (± 7,55) +++	44,4 (± 6,85)	66,9 (± 7,3) ++
	Avoidance/Flee	Frecuency	12,56 (± 1,29)	15,5 (± 1,84)	12,28 (± 0,99)	11,94 (± 1,02)
		Latency	9,32 (± 4,53)	7,16 (± 4,09)	5,93 (± 3,95)	1,85 (± 0,68)
		Time	90,61 (± 11,67)	69,92 (± 10,91)	73,25 (± 8,92)	62,52 (± 6,31)
Opponent (resident) mice	Threat	Frecuency	8,56 (± 0,87)	11,22 (± 0,79) +	5,95 (± 0,89)	9,21 (± 1,31) +
		Latency	5,58 (± 2,77)	8,34 (± 3)	30,03 (± 15,27)	27,54 (± 10,27)
		Time	50,68 (± 14,08)	83,47 (± 15,13)	29,6 (± 10,07)	48,23 (± 6,54)
	Attack	Frecuency	7,61 (± 1)	7,06 (± 1,15)	7,94 (± 0,78)	6,78 (± 1,08)
		Latency	27,69 (+ 8,1)	55,83 (+ 17,87)	21,22 (+ 13,14)	44,25 (+ 18,9)
		Time	34 (+ 5,03)	29,43 (+ 4,34)	41,12 (+ 5,32)	35,6 (+ 5,69)

Table 1. Behavioral categories evaluated during the first and fourth episode of social defeat in experimental and opponent mice. Defeated mice were classified as Low or High Submission according to the time spent in Defense/Submission behavior in the first defeat. + P < 0.05, ++ P < 0.01, +++ P < 0.001, significant difference with respect to IRSD Low Submission group.

Regarding the influence of the submissive profile on the behavioral measures obtained shortly after the last defeat exposure, the Student t test showed that, in comparison to High submissive mice, Low submissive mice performed more grooming behavior [t (34) = 3.44; p<0.001], spent more time engaged in this behavior [t (34) = -2.212; p<0.05], had a higher ISI [t (28) = 2.672; p<0.01] and showed a lower percentage of time [t (34) = 2.685; p<0.01] and percentage of entries [t (34) = 1.964; p<0.05] into the open arms of the EPM (see Table 2).

		IRSD Low Submission	IRSD High Submission
Splash test (grooming)	Frequency	25,12 (± 1,09)	20,11 (± 0,98) +++
	Latency	92,75 (± 7,82)	97,46 (± 7,7)
	Time	96,67 (± 5,42)	80,48 (± 4,94) +
Social interaction test	ISI	0,50 (± 0,11)	0,44 (± 0,02) ++
EPM (open arms)	Entries	15,5 (± 1,68)	18,33 (± 1,81)
	Latency	9,83 (± 3,35)	7,96 (± 3,92)
	Time	99,51 (± 9,02)	110,39 (± 10,37)
	Percentage of time	45 (± 3,9)	59,15 (± 3,55) ++
	Percentage of entries	43,36 (± 3,2)	53,57 (± 4,1) +
Hole-board test (dips)	Frequency	28,47 (± 3,69)	32,56 (± 4,69)
Tail suspension test	Time of immobility	8,41 (± 0,65)	8,96 (± 0,54)

Table 2. Measurements obtained in the behavioral tests performed short-term after IRSD in defeated mice classified as Low or High Submission according to the time spent in Defense/Submission behavior in the first defeat. + P < 0.05, ++ P < 0.01, +++ P < 0.001, significant difference with respect to IRSD Low Submission group.

The submissive profile also had an influence on resilience or vulnerability to the short- and long-term behavioral effects of IRSD (see Figure 3). When Low or High submissive mice were compared to the non-defeated control mice by means of ANOVA, the frequency of grooming [$F(2, 43) = 7.67$; $p<0.001$], ISI [$F(2, 43) = 9.631$; $p<0.001$] and percentage of time spent in the open arms of the EPM [$F(2, 43) = 3.966$; $p<0.05$] were significant. Only High submissive mice showed a reduction in the frequency of grooming in comparison to the control group ($p<0.05$) (Figure 3a). Both subgroups of defeated mice showed a reduction of ISI with respect to the control group, although the significance of the difference with respect to controls was higher in High submissive ($p<0.001$) than in Low submissive mice ($p<0.01$) (Figure 3b). In addition, as mentioned before, post-hoc comparison of the ANOVAs showed a difference between Low and High submissive mice in the frequency of grooming ($p<0.01$) (Figure 3a), ISI ($p<0.05$) (Figure 3b) and percentage of time in the open arms of the EPM ($p<0.05$) (Figure 3c).

Regarding the influence of the submissive profile on the long-term effects of IRSD on cocaine reward (see Figure 3d), the ANOVA showed a significant effect of the variable Days [$F(1, 39) = 16.483$; $p<0.001$] and the interaction Days X Group [$F(2, 39) = 3.860$; $p<0.05$]. The Bonferroni test revealed that only the High submissive mice developed CPP (significant difference between Pre- and Post-C, $p<0.001$).

Figure 3

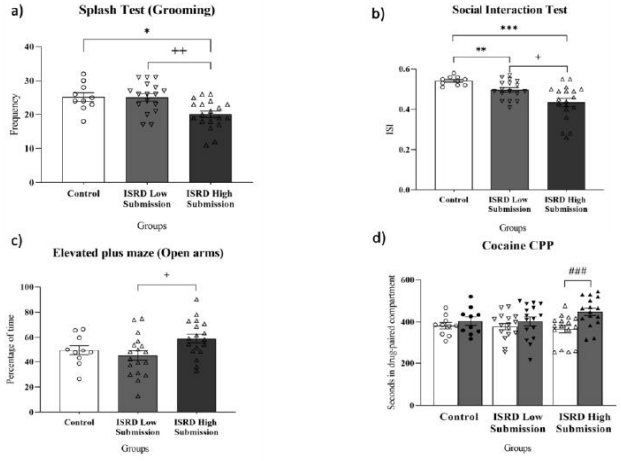

Figure 3. Behavioral profile during social defeat and resilience to the effects of IRSD. One group of early adolescent male mice was not exposed to stress (CONTROL, n = 10) and the other group was exposed to IRSD on PND 27, 30, 33 and 36 (n = 36). The behavior of mice during social defeat was evaluated and the group of defeated mice was divided into two subgroups, IRSD Low Submission and IRSD High Submission, according to their scores for time spent in Defense/Submission behavior during the first episode of defeat. **a)** Bars represent the mean (±SEM) number of times that the mice performed grooming behavior. * P < 0.05, ++ P < 0.01, significant difference with respect to the CONTROL group and ISRD High Submission group, respectively. **b)** Bars represent the mean (±SEM) ISI. **P < 0.01, ***P < 0.001, significant difference with respect to the CONTROL group; + P < 0.05, significant difference between Low and High Submissive groups. **c)** Bars represent the mean (±SEM) percentage of TOA on the EPM. + P < 0.05, significant difference between Low and High Submissive groups. **d)** Effects of IRSD on cocaine-induced CPP according to the behavioral profile of mice in the social defeats. All mice (CONTROL, IRSD Low Submission and IRSD High Submission groups) were conditioned with 1.5 mg/kg of cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, grey bars). ###P < 0.001, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.3. Behavioral Response in the Social Interaction Test and Resilience to the effects of IRSD (Cocaine CPP).

In order to evaluate resilience to the effects of IRSD in the social interaction test, defeated mice were separated into two groups according to their index of social interaction (ISI) score (below or above the median of the defeated group, which was 0.48): Low ISI or High ISI. A one-way ANOVA revealed a significant effect of the variable Group [$F(2,39)=41.420$, $p<0.001$]. Bonferroni post-hoc comparisons indicated that the Low ISI group was significantly different from the control and High ISI groups ($ps<0.001$) (Figure 4a). Thus, the mice in the High ISI defeated group were resilient to the impairing effects of IRSD on social interaction, since they did not engage in less social interaction.

Resilience to the impairing effects of IRSD on social interaction is associated with resilience to the effects of IRSD on cocaine reward. The ANOVA of the CPP data of the control group and the two groups of defeated mice classified in function of their ISI showed that the variable Days [$F(1,39)=18.541$; $p<0.001$] and the interaction Days x Group [$F(2,39)=4.177$; $p<0.05$] were significant. Post-hoc comparisons of the interaction revealed that only the Low ISI group displayed CPP (higher time spent in the drug-paired compartment in Post-C than in Pre-C, $p<0.001$). The control group of mice not exposed to defeat and the group of defeated mice that displayed a higher social interaction (High ISI group) did not develop CPP (see Figure 4b).

Figure 4

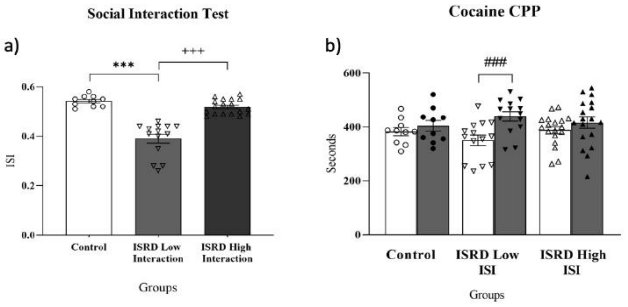

Figure 4. Behavior in the social interaction test and cocaine reward. One group of early adolescent male mice was not exposed to stress (CONTROL, n = 10) and the other group was exposed to IRSD on PND 27, 30, 33 and 36 (n = 36). **a)** Resilience to the short-term effects of IRSD on the social interaction test. The behavior of mice in the social interaction test was evaluated. The group of defeated mice was divided into two subgroups according to their index of social interaction (ISI), IRSD Low Interaction and IRSD High Interaction. Bars represent the mean (±SEM) ISI. ***P < 0.001, significant difference with respect to the CONTROL group; +++P < 0.001, significant difference with respect to the IRSD High Interaction group. **b)** Effects of IRSD on cocaine-induced CPP according to the behavioral profile of defeated mice in the social interaction test. All mice (CONTROL, IRSD Low ISI and IRSD High ISI groups) were conditioned with 1.5 mg/kg of cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and post-conditioning test (Post-C, grey bars). ### P < 0.001, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.4. Behavioral Response in the Splash Test and Resilience to Cocaine CPP

In order to evaluate resilience to the effects of IRSD in the splash test, the defeated mice were divided into two subgroups according to their scores of Frequency of grooming (below or above the median of the defeated group, which was 23 times), Low FG or High FG. One-way ANOVA showed a significant effect of the variable Group [F (2,39) = 29,036; p < 0.001]. Bonferroni post-hoc comparisons indicated that the Low FG IRSD group differed significantly from the control and High FG IRSD groups (ps< 0.001) (Figure 5a). When defeated mice were divided into two subgroups according to their scores in Time spent grooming (below or above the median of the defeated group, which was 85.7 seconds), Low TG or High TG, the one-way ANOVA showed a significant effect of the variable Group [F (2,39) = 24,411; p < 0.001] and Bonferroni post-hoc comparisons indicated that the Low TG IRSD group was significantly different from the control and High TG IRSD groups (ps< 0.001) (Figure 5b). The High FG and High TG groups did not differ from the control group; some defeated mice were resilient

to the effects of IRSD on the splash test and did not show a decrease in the frequency of grooming or in the time spent engaged in this behavior.

Resilience to the effects of IRSD on the splash test is associated with resilience to the long-term effects of IRSD on cocaine-induced CPP. The ANOVA of the CPP data of the control group and the two groups of defeated mice separated in function of their frequency of grooming showed that the variable Days [$F(1,39)= 14.873$, $p<0.001$] was significant. Simple Effects of the variable Days showed that the difference between Pre-C and Post-C was significant only in the Low FG group [$F(1,39)= 15.538$, $p<0.001$] (Figure 5c). The ANOVA of the CPP data of the control group and the two groups of defeated mice classified in function of the time spent grooming revealed that the variable Days [$F(1,39)= 18.406$, $p<0.001$] and the interaction Days x Group [$F(1,39)= 4.479$, $p<0.05$] were significant. Bonferroni post-hoc comparison of the interaction showed that only the Low TG IRSD group spent more time in the drug-paired compartment in Post-C than in Pre-C ($p< 0.001$). The control (non-defeated mice) and the High TG IRSD groups did not develop CPP (see Figure 5d). Thus, mice resilient to the effects of IRSD in the splash test were also resilient to the potentiation of cocaine reward induced by IRSD.

Figure 5

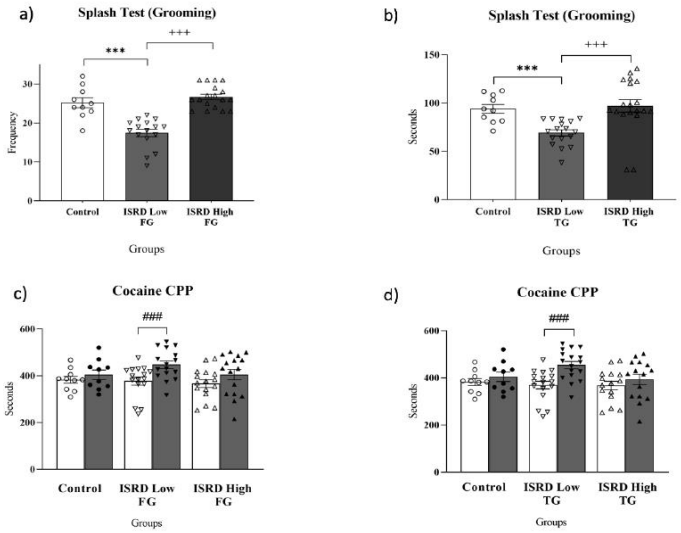

Figure 5. Behavior in the splash test and cocaine reward. One group of early adolescent male mice was not exposed to stress (CONTROL, n = 10) and the other group was exposed to IRSD on PND 27, 30, 33 and 36 (n = 36). **a)**

Resilience to the short-term effects of IRSD on the frequency of grooming behavior in the splash test. The group of defeated mice was divided into two subgroups according to their frequency of grooming (FG), IRSD Low FG and IRSD High FG. Bars represent the mean (±SEM) number of times that the mice performed grooming behavior. ***P < 0.001; +++ P < 0.001 significant difference vs. CONTROL and RSD High FG groups, respectively. **b)** Resilience to the short-term effects of IRSD on the time spent in grooming behavior in the splash test. The group of defeated mice was divided into two subgroups according to their time spent grooming (TG): IRSD Low TG and High TG. Bars represent the mean (±SEM) time in seconds that the mice spent in grooming behavior. ***P < 0.001; +++ P < 0.001 significant difference vs. the CONTROL and High TG groups, respectively. **c)** Effects of IRSD on cocaine-induced CPP according to the behavioral profile of defeated mice in the splash test (frequency of grooming). All mice (CONTROL, IRSD Low FG and IRSD High FG groups) were conditioned with 1.5 mg/kg of cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, grey bars). ###P < 0.001, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test. **d)** Effects of IRSD on cocaine-induced CPP according to the behavioral profile of defeated mice in the splash test (time in grooming). Mice (CONTROL, Low TG and High TG groups) were conditioned with cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre-C, white bars) and in the post-conditioning test (Post-C, grey bars). ###P < 0.001, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.5. Behavioral Response in the Elevated Plus Maze and Resilience to Cocaine CPP

Although IRSD did not induce effects in the EPM, in order to evaluate whether the behavioral profile of mice in the EPM was influencing resilience to the effects of IRSD on cocaine reward, defeated mice were divided into two subgroups according to their scores of Percentage of time in the open arms (below or above the median of the defeated group, which was 51%): Low or High %TOA. A one-way ANOVA revealed a significant effect of the variable Group [$F(2,39) = 26.223$; $p < 0.001$]. Bonferroni post-hoc comparisons indicated that the High %TOA ($p<0.05$) and Low %TOA ($p< 0.01$) groups were significantly different from the control group (Figure 6a). In addition, the Low and High %TOA groups also differed significantly ($p< 0.001$). Thus, no group of defeated mice displayed a behavioral profile similar to that of control mice.

However, the behavioral profile of mice in the EPM (Low or High %TOA) influenced resilience to the effects of IRSD on cocaine reward. The ANOVA of the CPP data for the Control and Low and High %TOA groups showed that the variable Days [$F(1,39) = 15.283$; $p< 0.001$] was significant. Simple effects of this variable showed that the effect of Days was significant only among the mice that spent a higher percentage of time in the open arms [$F(1,39) = 18.122$; $p< 0.001$]. Thus, the High %TOA spent more time in the drug-paired

compartment in Post-C than in Pre-C, while the control and the Low %TOA groups did not develop CPP (see Figure 6b).

Figure 6

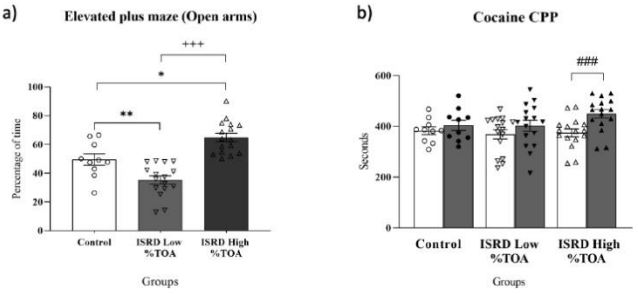

Figure 6. Behavior in the elevated plus-maze (EPM) and cocaine reward. One group of early adolescent male mice was not exposed to stress (CONTROL, n = 10) and the other group was exposed to IRSD on PND 27, 30, 33 and 36 (n = 36). **a)** Short-term effects of IRSD on the EPM. The group of defeated mice was divided into two subgroups according to the percentage of time they spent in the open arms (%TOA) of the EPM, IRSD Low %TOA and IRSD High %TOA. Bars represent the mean (±SEM) percentage of TOA. $* P < 0.05$, $** P < 0.001$, significant difference vs the control group. $+++ P < 0.001$, significant difference between the High and Low %TOA groups. **b)** Effects of IRSD on cocaine-induced CPP according to the behavioral profile of defeated mice in the EPM (percentage of time in open arms). All mice (CONTROL, IRSD Low % TOA and IRSD High % TOA groups) were conditioned with 1.5 mg/kg cocaine. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, white bars) and post-conditioning tests (Post-C, grey bars). $### P < 0.001$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

3.6. Behavioral Profile in the Hole Board and Tail Suspension Test did not predict Resilience to Cocaine CPP

The novelty-seeking (NS) profile of mice (Low vs High NS) and the behavioral profile of mice in the TST (Low or High time spent immobile) did no influenced resilience to the effects of IRSD on cocaine reward (see Supplementary Material and Supplementary Figure 1 and 2).

4. DISCUSSION

The present study demonstrated that exposure to IRSD is a useful paradigm to study the resilience to the behavioral short- and long-term effects of social stress in adolescent mice. Experience of IRSD during early adolescence induces depression-like symptoms short-term after defeats and increased the rewarding effects of cocaine at the initiation of adulthood, but some mice are resilient to these effects. In particular, mice with an active coping strategy during episodes of defeat (less defensive/submissive behaviors) are resilient to the effects of social defeat in the splash test and CPP paradigm. In addition, there are several behavioral traits associated to the resilience to the potentiating effect of IRSD on cocaine reward. Mice that are resilient to the depression-like symptoms induced by IRSD in the splash and social interaction tests, as well as mice that spend a lower percentage of time in the open arms of the EPM, are also resilient to the effects of IRSD on cocaine CPP.

4.1. Effects of IRSD on early adolescent mice.

Exposure to IRSD in early adolescence induces short- and long-term behavioral effects, including the development of depression-like symptoms in adolescent mice and enhanced sensitivity to the conditioned rewarding effects of cocaine in early adulthood. In line with these results, in a previous study in our laboratory we observed that mice exposed to IRSD in late adolescence also displayed a deficit of social interaction (Calpe-López et al., 2020; Calpe-López et al., 2022b) and a decrease in grooming in the splash test (Calpe-López et al., 2020; Calpe-López et al., 2022b and 2022c), that are indicative of depression-like behavior (Krishnan et al., 2007; Smolinsky et al., 2009). However, in the present study, an effect was not observed among mice exposed to IRSD during early adolescence when they performed the EPM (in accordance with the results obtained by Alves-Dos-Santos et al., 2020 with mice exposed to CSDS in early adolescence), or the tail suspension or hole-board tests. In contrast, we have previously observed that mice exposed to IRSD in late adolescence show anxiety-like symptoms (indicated by a decrease in the percentage of time spent in the open arms in the EPM) (Calpe-López et al., 2020; Calpe-López et al., 2022b and 2022c), an elevated stress responsivity (as suggested by a decrease of immobility in the tail suspension test) (Calpe-López et al., 2020; Calpe-López et al., 2022c) and a decrease in novelty-seeking in the hole board test (Calpe-López et al., 2022b and 2022c). These results indicate that early adolescent mice

experience social defeat less intensely than their older counterparts. In support of this idea, it has been observed that IRSD induces anxiety and cognitive deficits in late (García-Pardo et al., 2015) but not in early adolescent mice (Rodriguez-Arias et al., 2016). Furthermore, early adolescent mice were reported to show less defensive/submissive behavior (Montagud-Romero et al., 2017) and less avoidance/flee behavior (García-Pardo et al., 2015) during the first episode of defeat than late adolescent mice; in addition, aggressive mice confronted with early adolescent mice showed less aggressive behaviors than those confronted with late adolescent mice (García-Pardo et al., 2015; Montagud-Romero et al., 2017). In the same line, levels of corticosterone after social defeat are lower in early than in late adolescent mice (García-Pardo et al., 2015; Montagud-Romero et al., 2017). However, it is important to note that IRSD exposure induces long-term effects on cocaine reward in early adolescent mice, as previously observed in mice exposed to IRSD in late adolescence (Calpe-López et al., 2020; Calpe-López et al., 2022b). This result is also in line with several studies which have demonstrated that mice exposed to IRSD in early adolescence develop a preference for the compartment associated with a low dose of cocaine, which is ineffective in inducing CPP in non-stressed mice (Montagud-Romero et al., 2017; Rodriguez-Arias et al., 2017).

4.2. Resilience to the effects of IRSD is associated with the behavioral profile of mice during episodes of social defeat

Our results also demonstrate that some early adolescence mice are resilient to the effects of IRSD exposure, in accordance with what we have observed in late adolescent mice (Calpe-López et al., 2020). In particular, mice that spent less time in defense/submission behavior during the episodes of defeat were resilient to the short-term depression-like effects induced by IRSD and showed higher social interaction levels and more frequency of grooming in the splash test than mice which spent more time engaged in defense/submission behavior. In fact, there was a negative correlation between the time spent engaged in this behavior in the fourth defeat and the frequency of grooming. Although IRSD did not affect behavior in the EPM, in comparison to high submissive mice, low submissive mice spent a lower percentage of time in the open arms of the EPM, and there was a positive correlation between this measurement and the frequency of defense/submission in the first defeat. In addition, low submissive mice were resilient to the long-term effects of IRSD on cocaine reward and did not develop CPP. In fact,

there was a positive correlation between the time spent in defense/submission in the fourth defeat and the CPP score. In line with these results, we have previously observed that late adolescent mice showing low levels of submissive behavior are resilient to the effects of IRSD on cocaine CPP (Calpe-López et al., 2020). Considered together, these results indicate that the maintenance of an active coping strategy during episodes of social defeat is a consistent predictor of resilience to the effects of IRSD in adolescent mice.

4.3. Resilience to the short-term depression-like symptoms of IRSD predicts resilience to its long-term effects on cocaine reward

Among our experimental animals, there was a subgroup of defeated mice that was resilient to the impairing effects of IRSD on social interaction and that did not engage in less social interaction following social defeat. This result is in line with the observations of other studies with mice exposed to CSDS (Alves-dos-Santos et al., 2020) or to IRSD (Reguilón et al., 2022) during early adolescence. In addition, we observed that resilience to social avoidance is associated with subsequent resilience to the potentiation of cocaine reward, since defeated mice with an ISI similar to that of control mice did not acquire cocaine CPP. In relation to this, it is important to note that there was a quasi-significant negative correlation between ISI and CPP score, which suggests the importance of this variable in modulating the sensitivity of mice to the rewarding effects of cocaine. In line with this, early adolescent rats that were isolated between 5 and 25 days acquired CPP with a dose that was ineffective in socially housed rats (Cuesta et al., 2020; Starosciak et al., 2012; Zakharova et al., 2009). The association between resilience to the effects of defeat on ISI and on cocaine CPP has previously been observed in male mice exposed to IRSD in late adolescence (Ballestín et al., 2021; Calpe-López et al., 2020), indicating that resilience to defeat-induced social avoidance is a consistent predictor of resilience to the long-term effects of IRSD on the sensitivity of mice to cocaine reward. In contrast with our results, Reguilón et al. (2022) have reported that early adolescent mice classified as resilient based on their level of social interaction show a greater sensitivity to the rewarding effects of cocaine and develop CPP, which is the opposite effect to that observed in mice exposed to IRSD in late adolescence (Ballestín et al., 2021; Calpe-López et al., 2020). Indeed, we used an identical IRSD procedure and mice of the same strain, sex and age, so it is plausible that the divergent results are due to differences in the methodology employed in the social interaction test; in particular, the mouse used as an opponent when evaluating social

avoidance. While Reguilón et al. (2022) used a mouse of the same strain (C57BL/6J), we used a mouse of the OF1 strain (as in the defeat episodes). In this context, it was reported that when the target in the social interaction test was a mouse of the C57BL/6J strain, both susceptible and resilient mice of the same strain spent more time in the interaction zone than when the aggressive opponent was of the CD1 strain, although the social interaction was significantly higher in resilient than in susceptible mice (Han et al., 2014).

We have also observed that some defeated mice remained resilient to the effects of IRSD on the Splash Test; i.e., they did not display a reduction in the frequency of grooming. Given that this reduction in the time and frequency of grooming has been interpreted as anhedonia (Brachman et al., 2016; de Souza, et al., 2019), our results suggest that some mice are resilient to the depression-like effects of IRSD. Similar results have been reported by Alves-Dos-Santos et al. (2020), who observed that approximately half of the mice exposed to CSDS in early adolescence were resilient to the decrease in sucrose preference. In the present study, resilience to the short-term effects of IRSD on the frequency of and time spent in grooming predicts subsequent resilience to cocaine reward; only vulnerable mice displaying reduced grooming behavior acquired CPP three weeks after the last episode of defeat. The same results were reported previously in mice exposed to IRSD in late adolescence (Calpe-López et al., 2020).

Although the response of defeated mice in both the social interaction and splash tests was predictive of their subsequent resilience or vulnerability to cocaine reward, we did not detect correlations between ISI and the measurements of grooming. This suggests that these behavioral tests measure unrelated behaviors. In accordance with our results, an absence of correlation between social avoidance and the decrease in sucrose preference induced by exposure to CSDS in early adolescent mice has been reported by Alves-Dos-Santos et al. (2020). Similarly, in a previous study, we did not observe correlations between ISI and grooming in mice exposed to IRSD in late adolescence (Calpe-López et al., 2020).

4.4. Behavioral profile of defeated mice in the EPM predicts Resilience to the long-term effects of IRSD on cocaine reward

Although exposure to IRSD in early adolescence did not induce effects in the EPM, we saw that the behavioral profile of defeated mice in this test predicted their subsequent sensitivity to cocaine reward. After segregating the defeated animals into two subpopulations according to the percentage of time spent in the open arms of the EPM, we observed that mice which spent a lower percentage of time in the open arms were resilient to the long-term effects of IRSD and did not acquire cocaine CPP, while defeated ice that spent a higher percentage of time in the open arms displayed enhanced vulnerability to the rewarding effects of cocaine and developed CPP. These results may seem surprising given the close association between anxiety and cocaine use disorders (Vorspan et al., 2015), since a low percentage of time spent in the open arms of the EPM is usually considered indicative of anxiety (Campos et al., 2013). However, we have also previously observed that mice exposed to IRSD in late adolescence and which spent a lower percentage of time in the open arms of the EPM did not develop CPP (Calpe-López et al., 2020). As we reported in our previous study, the EPM test not only reveals an anxious state; the higher percentage of time spent in the open arms by the mice that developed CPP might indicate a pre-existing impulsive phenotype (Gass et al., 2014) that predisposes them to be more vulnerable to the effects of cocaine. Furthermore, the EPM entails a conflict between two natural tendencies: the motivation to remain in the closed arms (associated with safety) and the motivation to explore the open arms, which could be a potential danger or threat (Ennaceur and Chazot, 2016). Indeed, in a recent study carried out in our laboratory we demonstrated a positive correlation between the percentage of time spent in the open arms and the distance travelled in the EPM (Calpe-López et al., 2022b), thus supporting the idea that a motivation to explore predominates among mice that spend a higher percentage of time in the open arms. Conversely, mice that spent a lower percentage of time in the open arms would prefer to feel safe than to explore. In support of this hypothesis, we observed a negative correlation between immobility in the tail suspension test and the percentage of time spent in open arms. In addition, behavior during the first episode of defeat was also related with this measure. The percentage of time spent in the open arms correlated positively with the frequency of defense/submission, and negatively with avoidance/flee behavior. Thus, we interpret that defeated mice which are resilient to the long-term effects of IRSD on cocaine reward are those that actively avoid the open arms to stay safe from other potential threats after experiencing attack by an opponent.

5. CONCLUSION

The present study demonstrates that there are several behavioral traits that predict vulnerability or resilience to the effects of IRSD in early adolescent mice. Highly submissive mice are more vulnerable to the effects of social stress, as they develop depression-like symptoms shortly after defeat and exhibit a long-term enhanced vulnerability to the rewarding effects of cocaine. Conversely, the maintenance of an active coping strategy during episodes of social defeat (mainly characterized by low levels of submissive behavior) predicts resilience to the short-term depression-like effects of IRSD in the social interaction and splash tests and to the long-term effects of IRSD on cocaine reward. Furthermore, resilient defeated mice who showed an absence of social avoidance and unaltered levels of grooming were also resilient to the development of cocaine CPP. The behavioral profile in the EPM - characterized by a lower percentage of time spent in the open arms - is also related with resilience to the effects of IRSD on cocaine CPP. Indeed, all these variables predict resilience to the effects of IRSD in late adolescent mice. Conversely, the behavioral profile of mice defeated in early adolescent in the hole board or tail suspension tests is associated with neither resilience nor vulnerability to the long-term effects of IRSD, in contrast with the influence of these variables on mice exposed to defeat in late adolescence, which has previously been reported. From a translational view, our results suggest that behavioral interventions that increase the pro-active response of adolescents exposed to bullying may enhance their resilience to the negative consequences of this stressful experience and prevent the development of depressive and addictive disorders.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1507028485045).

Supplementary Material

Contributors: MAA and MPG-P contributed to the conception and design of the study. CC-L and MAM-C, performed the experiments, organized the databases and performed the statistical analyses. CC-L and MPG-P wrote the first draft of the manuscript. MAA wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of interest statement

Conflict of Interest: No conflict declared.

26th September 2022

Dear Editor,

Please find enclosed the manuscript ***Resilience to the Short- and Long-term Behavioral Effects of Intermittent Repeated Social Defeat in Adolescent Male Mice*** by Claudia Calpe-López, Maria A Martínez-Caballero, Maria P García-Pardo and Maria A Aguilar.

This work has not been published previously and is not under consideration for publication elsewhere. All listed authors have contributed significantly to the manuscript and have given consent for their names to be included in the manuscript. Moreover, the authors have no possible conflict of interest in the carrying out and reporting of this research. 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 Directive 2010/63/EU of the European Parliament and of the council of September 22, 2010 on the protection of animals used for scientific purposes, including the advance principles of the 3R guidelines (i.e., refinement, replacement, reduction of animal use).

We look forward to hearing from you,

Sincerely yours,

Dra. Maria A Aguilar

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Figures and Tables

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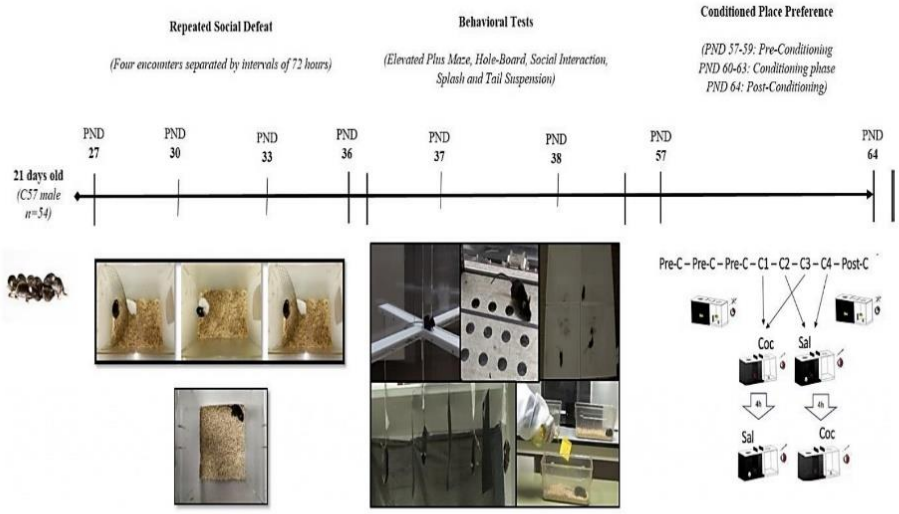
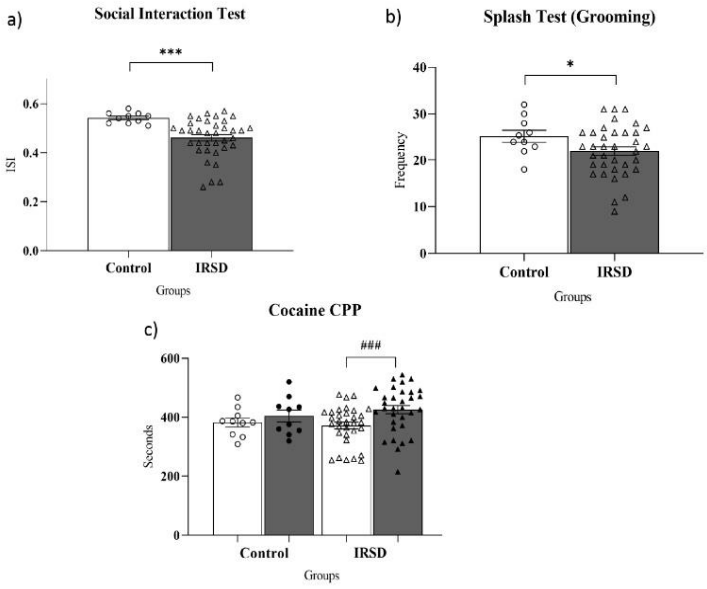


Figure 2



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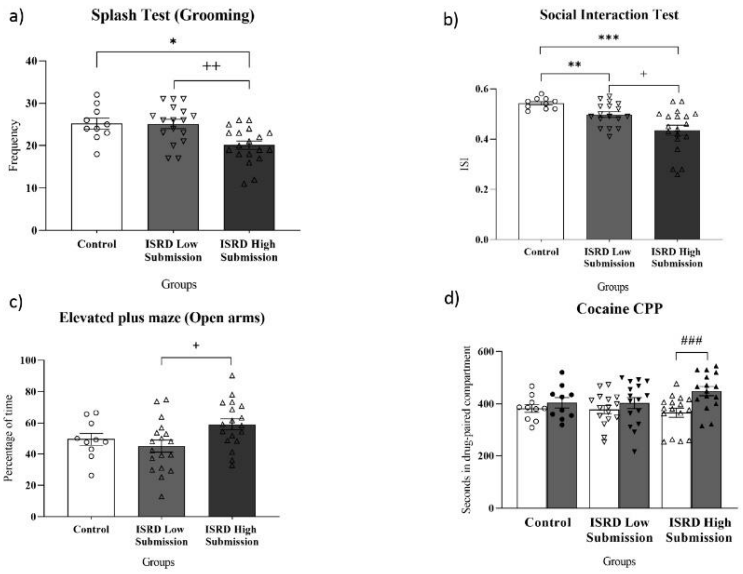


Figure 4

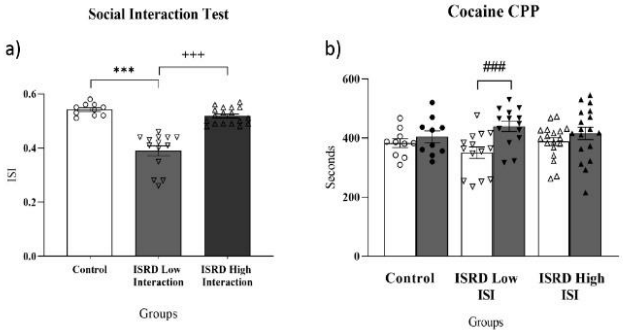
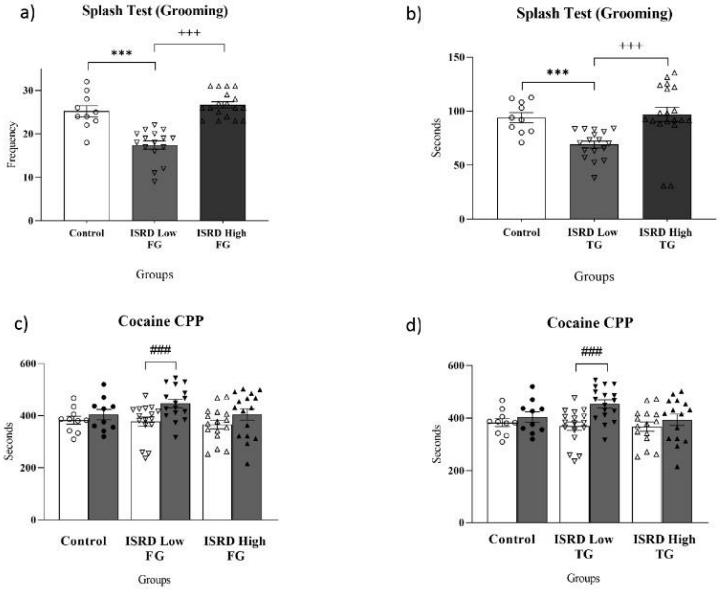


Figure 5



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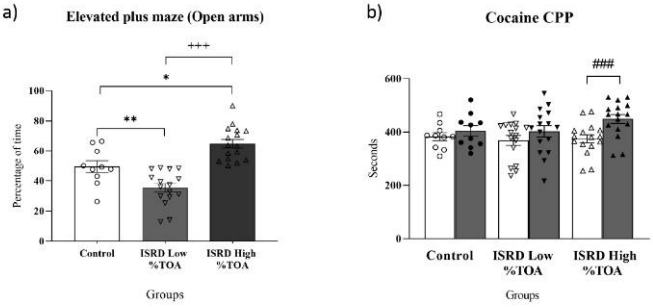
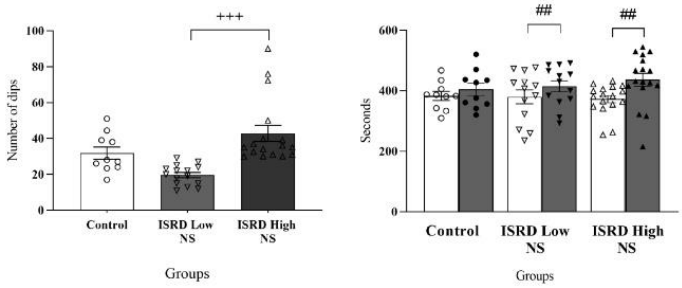


Supp. Figure 1 a) **Hole Board Test (Novelty Seeking)** b) **Cocaine CPP**



Suppl. Figure 2 a) **Tail Suspension Test (Immobility)** b) **Cocaine CPP**

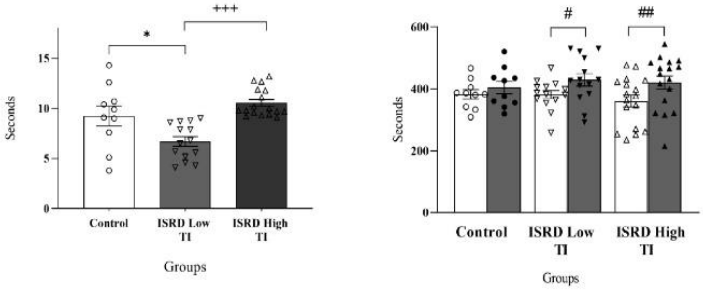


Table 1.

			1st SD		4th SD	
			IRSD Low Submission	IRSD High Submission	IRSD Low Submission	IRSD High Submission
Experimental (intruder) mice	Defense/Submission	Frecuency	7,55 (± 1)	14,68 (± 0,95) +++	8,61 (± 0,95)	12,44 (± 1,21) ++
		Latency	28,47 (± 6,8)	9,79 (± 2,57) ++	19,02 (± 4,41)	24,93 (± 8,66)
		Time	29,61 (± 3,73)	79,72 (± 7,55) +++	44,4 (± 6,85)	66,9 (± 7,3) ++
	Avoidance/Flee	Frecuency	12,56 (± 1,29)	15,5 (± 1,84)	12,28 (± 0,99)	11,94 (± 1,02)
		Latency	9,32 (± 4,53)	7,16 (± 4,09)	5,93 (± 3,95)	1,85 (± 0,68)
		Time	90,61 (± 11,67)	69,92 (± 10,91)	73,25 (± 8,92)	62,52 (± 6,31)
Opponent (resident) mice	Threat	Frecuency	8,56 (± 0,87)	11,22 (± 0,79) +	5,95 (± 0,89)	9,21 (± 1,31) +
		Latency	5,58 (± 2,77)	8,34 (± 3)	30,03 (± 15,27)	27,54 (± 10,27)
		Time	50,68 (± 14,08)	83,47 (± 15,13)	29,6 (± 10,07)	48,23 (± 6,54)
	Attack	Frecuency	7,61 (± 1)	7,06 (± 1,15)	7,94 (± 0,78)	6,78 (± 1,08)
		Latency	27,69 (± 8,1)	55,83 (± 17,87)	21,22 (± 13,14)	44,25 (± 18,9)
		Time	34 (± 5,03)	29,43 (± 4,34)	41,12 (± 5,32)	35,6 (± 5,69)

Table 2.

		IRSD Low Submission	IRSD High Submission
Splash test (grooming)	Frequency	25,12 (± 1,09)	20,11 (± 0,98) +++
	Latency	92,75 (± 7,82)	97,46 (± 7,7)
	Time	96,67 (± 5,42)	80,48 (± 4,94) +
Social interaction test	ISI	0,50 (± 0,11)	0,44 (± 0,02) ++
EPM (open arms)	Entries	15,5 (± 1,68)	18,33 (± 1,81)
	Latency	9,83 (± 3,35)	7,96 (± 3,92)
	Time	99,51 (± 9,02)	110,39 (± 10,37)
	Percentage of time	45 (± 3,9)	59,15 (± 3,55) ++
	Percentage of entries	43,36 (± 3,2)	53,57 (± 4,1) +
Hole-board test (dips)	Frequency	28,47 (± 3,69)	32,56 (± 4,69)
Tail suspension test	Time of immobility	8,41 (± 0,65)	8,96 (± 0,54)

Suppl. Table 1

		Splash FREQ	Splash LAT	Splash TIME	Hole-B DIPS	Soc Int ISI	Tail S Imm	EPM EOA	EPM LatOA	EPM TimeOA	EPM %TOA	EPM %EOA	CPP Score
Splash FREQ	Pearson	1	-0.344*	0.649**	-0.069	0.222	-0.075	-0.099	0.099	0.056	-0.095	0.064	-0.186
	Significance		0.019	0.000	0.649	0.138	0.625	0.514	0.514	0.713	0.530	0.670	0.226
	N	46	46	46	46	46	45	46	46	46	46	46	44
Splash LAT	Pearson	-0.344*	1	-0.099	-0.046	0.120	-0.128	-0.012	-0.089	-0.122	0.075	0.009	0.094
	Significance	0.019		0.513	0.761	0.426	0.400	0.939	0.554	0.419	0.619	0.955	0.544
	N	46	46	46	46	46	45	46	46	46	46	46	44
Splash TIME	Pearson	0.649**	-0.099	1	-0.120	0.084	0.015	-0.086	0.249	-0.128	-0.184	-0.032	0.013
	Significance	0.000	0.513		0.426	0.577	0.924	0.570	0.095	0.395	0.220	0.832	0.934
	N	46	46	46	46	46	45	46	46	46	46	46	44
Hole-B DIPS	Pearson	-0.069	-0.046	-0.120	1	-0.060	-0.230	0.045	0.103	-0.310*	-0.261	-0.213	0.158
	Significance	0.649	0.761	0.426		0.690	0.129	0.769	0.495	0.036	0.079	0.156	0.306
	N	46	46	46	46	46	45	46	46	46	46	46	44
Soc Int ISI	Pearson	0.222	0.120	0.084	-0.060	1	-0.107	0.078	-0.040	-0.080	-0.089	0.102	-0.286
	Significance	0.138	0.426	0.577	0.690		0.485	0.608	0.791	0.597	0.557	0.499	0.060
	N	46	46	46	46	46	45	46	46	46	46	46	44
Tail S Imm	Pearson	-0.075	-0.128	0.015	-0.230	-0.107	1	-0.040	-0.012	-0.066	-0.331*	-0.052	-0.016
	Significance	0.625	0.400	0.924	0.129	0.485		0.792	0.938	0.668	0.026	0.733	0.918
	N	45	45	45	45	45	45	45	45	45	45	45	43
EPM EOA	Pearson	-0.099	-0.012	-0.086	0.045	0.078	-0.040	1	0.086	0.306*	0.288	0.478**	0.031
	Significance	0.514	0.939	0.570	0.769	0.608	0.792		0.569	0.039	0.052	0.001	0.840
	N	46	46	46	46	46	45	46	46	46	46	46	44
EPM LatOA	Pearson	0.099	-0.089	0.249	0.103	-0.040	-0.012	0.086	1	-0.272	0.027	0.147	0.090
	Significance	0.514	0.554	0.095	0.495	0.791	0.938	0.569		0.068	0.857	0.331	0.562
	N	46	46	46	46	46	45	46	46	46	46	46	44
EPM TimeOA	Pearson	0.056	-0.122	-0.128	-0.310*	-0.080	-0.066	0.306*	-0.272	1	0.338*	0.269	-0.202
	Significance	0.713	0.419	0.395	0.036	0.597	0.668	0.039	0.068		0.021	0.070	0.189
	N	46	46	46	46	46	45	46	46	46	46	46	44
EPM %TOA	Pearson	-0.095	0.075	-0.184	0.261	-0.089	-0.331*	0.288	0.027	0.338*	1	-0.453**	0.119
	Significance	0.530	0.619	0.220	0.079	0.557	0.026	0.052	0.857	0.021		0.002	0.441
	N	46	46	46	46	46	45	46	46	46	46	46	44
EPM %EOA	Pearson	0.064	0.009	-0.032	0.213	0.102	-0.052	0.478**	0.147	0.269	-0.453**	1	0.063
	Significance	0.670	0.955	0.832	0.156	0.499	0.733	0.001	0.331	0.070	0.002		0.684
	N	46	46	46	46	46	45	46	46	46	46	46	44
CPP Score	Pearson	-0.186	0.094	0.013	0.158	-0.286	-0.016	0.031	0.090	-0.202	0.119	0.063	1
	Significance	0.226	0.544	0.934	0.306	0.060	0.918	0.840	0.562	0.189	0.441	0.684	
	N	44	44	44	44	44	43	44	44	44	44	44	44

Suppl. Table 2

		1st Time Avoidance/ Flight	1st Freq Defense/ Submission	1st Lat Defense/ Submission	1st Time Defense/ Submission	1st Freq Threat	4th Time Defense/ Submission	4th Time Defense/ Submission	4th Freq Threat
1st Time Avoidance/ Flight	Pearson	1	-0.521**	0.504**	-0.358*	-0.194	-0.346	-0.307	-0.017
	Significance		0.002	0.003	0.044	0.288	0.053	0.088	0.927
	N	32	32	32	32	32	32	32	32
1st Freq Defense/ Submission	Pearson	-0.521**	1	-0.565**	0.786**	0.636**	0.200	0.413*	0.132
	Significance	0.002		0.001	0.000	0.000	0.272	0.019	0.471
	N	32	32	32	32	32	32	32	32
1st Lat Defense/ Submission	Pearson	0.504**	-0.565**	1	-0.402*	-0.224	-0.178	-0.293	0.027
	Significance	0.003	0.001		0.022	0.217	0.330	0.103	0.884
	N	32	32	32	32	32	32	32	32
1st Time Defense/ Submission	Pearson	-0.358*	0.786**	-0.402*	1	0.520**	0.256	0.501**	0.192
	Significance	0.044	0.000	0.022		0.002	0.157	0.003	0.292
	N	32	32	32	32	32	32	32	32
1st Freq Threat	Pearson	-0.194	0.636**	-0.224	0.520**	1	0.211	0.416*	0.249
	Significance	0.288	0.000	0.217	0.002		0.246	0.018	0.170
	N	32	32	32	32	32	32	32	32
4th Freq Defense/ Submission	Pearson	-0.346	0.200	-0.178	0.256	0.211	1	0.573**	0.278
	Significance	0.053	0.272	0.330	0.157	0.246		0.000	0.101
	N	32	32	32	32	32	36	36	36
4th Time Defense/ Submission	Pearson	-0.307	0.413*	-0.293	0.501**	0.416*	0.573**	1	0.597**
	Significance	0.088	0.019	0.103	0.003	0.018	0.000		0.000
	N	32	32	32	32	32	36	36	36
4th Freq Threat	Pearson	-0.017	0.132	0.027	0.192	0.249	0.278	0.597**	1
	Significance	0.927	0.471	0.884	0.292	0.170	0.101	0.000	
	N	32	32	32	32	32	36	36	36

Suppl. Table 3

		Splash FREQ	Splash LAT	Splash TIME	Hole-B DIPS	Soc Int ISI	Tail S Imm	EPM EOA	EPM LatOA	EPM TOA	EPM %TOA	EPM %EOA	CPP Score
1st Time Avoidance /Flight	Pearson	0.000	-0.039	0.216	0.177	-0.049	-0.012	-0.138	0.140	-0.280	-0.353*	-0.190	0.069
	Sig.	0.856	0.832	0.235	0.332	0.791	0.948	0.451	0.446	0.120	0.048	0.296	0.718
	N	32	32	32	32	32	31	32	32	32	32	32	30
1st Freq Defense/ Submission	Pearson	-0.153	0.123	-0.109	-0.136	-0.229	0.004	0.323	0.162	0.388*	0.490**	0.301	0.132
	Sig.	0.402	0.503	0.554	0.457	0.207	0.982	0.071	0.375	0.028	0.004	0.094	0.487
	N	32	32	32	32	32	31	32	32	32	32	32	30
1st Lat Defense/ Submission	Pearson	0.071	-0.036	0.096	0.371*	0.166	-0.066	-0.056	0.056	-0.280	-0.319	-0.032	-0.065
	Sig.	0.701	0.845	0.601	0.037	0.363	0.724	0.759	0.761	0.121	0.075	0.864	0.731
	N	32	32	32	32	32	31	32	32	32	32	32	30
1st Time Defense/ Submission	Pearson	-0.269	0.265	-0.281	0.028	-0.228	0.095	0.132	-0.070	0.194	0.291	0.078	0.099
	Sig.	0.137	0.143	0.119	0.878	0.210	0.611	0.470	0.705	0.287	0.106	0.670	0.603
	N	32	32	32	32	32	31	32	32	32	32	32	30
1st Freq Threat	Pearson	-0.081	0.288	0.073	0.027	-0.020	-0.118	0.190	0.263	0.052	0.231	-0.020	0.040
	Sig.	0.661	0.110	0.691	0.882	0.912	0.526	0.297	0.147	0.779	0.204	0.912	0.833
	N	32	32	32	32	32	31	32	32	32	32	32	30
4th Freq Defense/ Submission	Pearson	-0.284	0.150	-0.111	0.099	-0.022	0.175	-0.065	0.154	-0.184	0.096	0.095	0.339*
	Sig.	0.093	0.382	0.519	0.567	0.899	0.314	0.707	0.369	0.283	0.577	0.580	0.050
	N	36	36	36	36	36	35	36	36	36	36	36	34
4th Time Defense/ Submission	Pearson	-0.409*	0.276	-0.308	-0.012	-0.157	0.143	0.000	0.072	-0.240	0.032	0.003	-0.047
	Sig.	0.013	0.104	0.068	0.944	0.360	0.412	0.999	0.677	0.159	0.852	0.987	0.791
	N	36	36	36	36	36	35	36	36	36	36	36	34
4th Freq Threat	Pearson	-0.108	-0.054	-0.016	0.299	-0.085	0.065	-0.007	0.004	-0.401*	0.099	-0.044	-0.028
	Sig.	0.529	0.753	0.927	0.077	0.620	0.711	0.968	0.981	0.015	0.564	0.800	0.874
	N	36	36	36	36	36	35	36	36	36	36	36	34

Chapter 4

Claudia Calpe-López, María Ángeles Martínez-Caballero, María Pilar García-Pardo, María A. Aguilar. Modulation of the effects of alcohol, cannabinoids and psychostimulants by novelty-seeking trait. In: Methods for Preclinical Research in Addiction, Neuromethods, vol. 174, María A. Aguilar (ed.), https://doi.org/10.1007/978-1-0716-1748-9_8,

Chapter 4

Modulation of Effects of Alcohol, Cannabinoids, and Psychostimulants by Novelty-Seeking Trait

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Abstract

A main challenge for addiction research is to identify the factors that predispose individuals to drug addiction. Novelty- or sensation-seeking is a personality trait which partly explains individual differences in vulnerability to addiction. The hole board test is an animal model of novelty-seeking behavior used to study the influence of this behavioral trait in sensitivity to the rewarding effects of drugs. In our laboratory we have also used the hole board test to determine how the novelty-seeking phenotype modulates the long-lasting effects of adolescent exposure to psychostimulant drugs, alcohol, or cannabinoids on the subsequent rewarding properties of drugs of abuse in the conditioned place preference (CPP) paradigm. In this chapter we provide a detailed description of the hole board and CPP procedures and of different types of drug pretreatments. First, adolescent mice are classified as high novelty-seeking (HNS) or low novelty-seeking (LNS) according to the number of dips they perform in the hole board test. Next, the mice are exposed to repeated injections of cocaine, MDMA, alcohol or the cannabinoid agonist WIN 55212-2 during the adolescent period. Finally, the acquisition, extinction and reinstatement of drug-induced CPP is evaluated in HNS and LNS mice. Our results suggest that the high novelty-seeking endophenotype is a marker of enhanced susceptibility to the effects of environmental variables that increase the risk of drug addiction, such as adolescent drug exposure or stressful experiences. New knowledge concerning the contribution of novelty-seeking to the development of addiction is likely to contribute to the development of better preventive and treatment strategies for this disorder.

Key words Adolescence, Alcohol, Cannabinoids, Cocaine, Hole board, MDMA, Mice, Novelty-seeking

1 Introduction

1.1 The Novelty-Seeking Trait

A main challenge for addiction research is to identify the factors that predispose individuals to drug addiction in order to develop better preventive and treatment strategies [1]. Besides environmental factors, including exposure to the drug itself and stress, there are personality traits or vulnerable phenotypes (*see* Fig. 1) that may be present before the first experience of the drug and which explain

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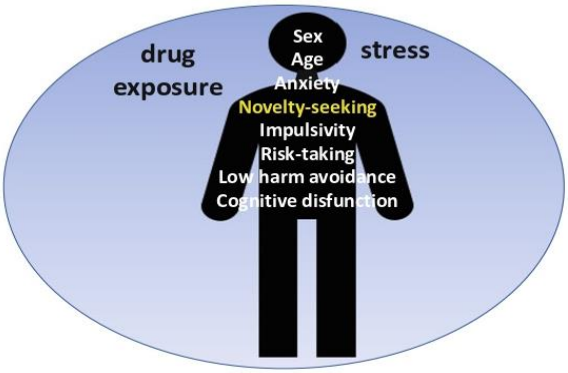

Environmental and individual factors associated with vulnerability to drug addiction

Fig. 1 Environmental and individual factors associated with vulnerability to drug addiction

individual differences in sensitivity to the rewarding effects of drugs and vulnerability to develop addiction [2].

Endophenotypes are heritably determined quantitative traits associated with a disorder, and which connect genotype (predisposing genes) and phenotype (clinical symptoms of the disorder) [3]. Such traits are found in affected individuals, manifest themselves with and without the active illness/diagnosis, cosegregate according to the disorder within families, and are more prevalent among nonaffected family members than in the general population [4]. The high novelty-seeking profile, as well as cognitive dysfunction and anxious-impulsive personality traits [3], may be considered as an endophenotype for drug dependence.

Novelty- or sensation-seeking is a personality trait defined as a tendency to pursue novel and intense emotional sensations and experiences [5]. Individuals with a high novelty-seeking profile like taking risks and seeking new environments and situations to increase their level of arousal and intensify their experiences [6]. The recently deceased Marvin Zuckerman was the first author who defined the term of novelty- or sensation-seeking, and developed the most widely used sensation seeking scale (Zuckerman's Sensation Seeking Scale) [6]. Cloninger extended the work of Zuckerman and developed the Cloninger's Tridimensional Personality Questionnaire (TPQ), which also measured the novelty-seeking trait [7]. Other authors have extended our understanding of the novelty-seeking phenotype and its relationship with drug use disorders. Novelty-seeking is a multifaceted behavioral construct that includes personality characteristics usually displayed by individuals at a higher risk of developing drug addiction, such as thrill-seeking,

novelty-preference, risk-taking, and harm avoidance [2, 6, 8–13]. There is a great deal of evidence of the relationship between the novelty-seeking trait and drugs of abuse. A high novelty-seeking profile predicts increased drug use during adolescence [2, 3, 8, 14–18]. For example, high-sensation seekers are more likely to experiment with tobacco, alcohol, marihuana and MDMA [19], display greater incentive motivation to self-administer amphetamine in a controlled laboratory study [20], and are more responsive to the positive subjective effects of this drug in various scales, such as the Profile of Mood States (POMS) and Visual Analogue Scale (VAS) [20, 21]. Furthermore, drug use itself enhances the novelty-seeking trait [22] and both novelty- and drug-seeking behavior are mediated by the mesolimbic dopaminergic system [23].

1.2 Adolescence and Novelty-Seeking

Adolescence is a critical developmental period characterized by hormonal changes and neuroplasticity, especially in the dopaminergic mesocorticolimbic areas, that contribute to the maturation of the central nervous system [24, 25]. In comparison to adults, adolescent subjects exhibit lower dopamine release in basal conditions and hypersensitivity of the dopaminergic striatal system to pharmacological or environmental stimuli (including drugs of abuse and exposure to novel environments) [26–30]. In addition, the prefrontal cortical system, related with planning, evaluation of consequences, decision-making, and inhibitory control of behavior, is still immature [31, 32]. Thus, the adolescent brain operates in a promotivational state due to a limited inhibitory capacity, poor regulatory control, and dopaminergic hyperactivity in the nucleus accumbens and amygdala [33].

Related with these changes in the dopaminergic systems [24, 34], adolescents display several personality characteristics that may explain why it is most likely that drug use begins during adolescence, such as impulsiveness, a natural drive to search for novel stimuli and sensations, willingness to take risks, low novelty- and risk-induced anxiety, and emotional liability [13, 31, 35, 36]. Furthermore, at this developmental stage the cognitive control and harm-avoidance systems are ineffective for limiting drug consumption [37, 38], and so the rewarding effects of addictive drugs predominate over their aversive effects [39, 40], thus augmenting the propensity to take drugs.

Although there is a positive correlation between early drug-taking and the development of drug dependence in adulthood [41–43], not all drug users progress to drug abuse, dependence, and addiction. Longitudinal studies point to an association between the novelty-seeking trait and the initiation of drug use [2, 44] and young adults with a high novelty-seeking profile show a greater sensitivity to the physiological and psychological effects of drugs in comparison with low novelty seekers [20, 21, 45–49]. In any case,

the transition from voluntary to compulsive drug use that characterizes addiction depends on the combination of multiple factors, including the novelty-seeking trait.

1.3 Animal Models of Novelty-Seeking

Novelty-seeking is a complex behavior that involves the detection of changes in the environment (cognition), the natural tendency to approach and explore novel objects, and the individual's stress responsiveness [50]. Although rodents show an innate preference for novel objects over familiar ones [51], the behavior of an animal in a novel environment is conditioned by the interaction of several factors, including activity, motivation to explore and fear/anxiety [52, 53].

Different procedures allow the screening of novelty-seeking behavior in rats and mice, among which two of the most popular (and contrasting) are the inescapable vs. free choice novelty models. It is important to note that the procedure employed to measure novelty-seeking trait can influence the results obtained (*see* Table 1), particularly regarding the relationship between this endophenotype and vulnerability to drugs of abuse (*see* Table 2).

1.3.1 Inescapable Novelty Models

This approach, frequently known as novelty responding, is the most used to classify animals as high or low responders according to their locomotor activity in a new inescapable environment. In these procedures, animals are confined to the new place, without allowing them the freedom of choice to explore the novel object or environment. These models of novelty-seeking are artificial and have been criticized because it is unclear whether the locomotor reactivity displayed by rodents represents an escape or an exploratory behavior.

Locomotor Reactivity in a Novel Open Field Environment

This test was the first animal model of individual differences in stress responsiveness to a novel inescapable environment [54]. Rodents are placed in a novel open field environment and the activity, distance traveled and rears are recorded over different periods of time (usually between 10 min and 2 h). Animals that exhibit high levels of novelty-induced locomotor reactivity are categorized as High Responders (HR) and those showing lower levels as Low Responders (LR).

A relationship between novelty-induced locomotor activity and drug-taking behaviors has been observed in the HR–LR model [55–57]. For example, HR rats exhibit high rates of behavioral sensitization to psychostimulant drugs and self-administer these drugs more readily than LR rats [54, 56]. In line with this, Davis et al. [58] suggested that "the HR–LR trait may tap into the broad dimension of behavioral disinhibition vs. behavioral control—a dimension that has been implicated in the vulnerability versus resilience to numerous psychiatric and addictive disorders."

Table 1
Levels of novelty-seeking behavior in adolescent and young adult mice of both sexes in different paradigms of novelty-seeking

PARADIGM OF NOVELTY SEEKING	MICE			
	MALE		FEMALE	
Inescapable (locomotor activity)	ADOLESCENT	YOUNG ADULT	ADOLESCENT	YOUNG ADULT
NOVELTY-REACTIVITY (10 min in environment)				
NOVELTY-HABITUATION (1h in environment)				
Free choice				
NOVEL OBJECT RECOGNITION TASK				
NOVEL ENVIRONMENT TEST				
HOLE BOARD				

Level of novelty seeking: low — high

Activity in the Wheel Running Procedure

In this model, rodents are classified as HR and LR according to their motor response to novelty on the first day they are placed on a running wheel [59]. It is important to note that the next day both groups showed a similar locomotor activity.

Activity in an Exploration Box Test

In this test, considered an indicator of inquisitive and inspective exploration, animals are placed in a box containing three unknown objects and one familiar food pellet (in the same location) on 5 consecutive days. According to the locomotor and rearing activity the animals display during exploration of the three unfamiliar objects, they are classified as high or low exploratory activity (HE or LE, respectively) [60, 61].

1.3.2 Free-Choice Models

The paradigms that evaluate preference for novel objects or environments in a free-choice procedure allow animals to choose or to reject novelty [2, 12, 62–64]. This is why they are currently considered a better measure of novelty-seeking and more suitable for categorizing the novelty-seeking trait.

The Novel Environment Test

This test is performed in a rectangular box divided by a partition into two compartments of equal size but different colors that are connected by an opening with a removable guillotine-type door located at floor level in the center of the partition. In order to establish familiarity with an environment, animals are placed in one of the compartments that they may explore freely with no access to the other compartment. In our laboratory, animals undergo a single habituation session of 15 min [65, 66], but some authors carry out more sessions [67]. After habituation, the guillotine-type door is raised, allowing free access to and exploration of both compartments for 20 min (test session). Usually the behavior of the animal is videotaped under red light and the number of transitions between the two compartments, the latency for

Table 2
Acquisition of CPP induced by cocaine (1 mg/kg) according to the level of novelty-seeking shown by adolescent and young adult mice of both sexes in different paradigms of novelty-seeking

PARADIGM OF NOVELTY SEEKING	MICE			
	MALE		FEMALE	
Inescapable (locomotor activity)	ADOLESCENT	YOUNG ADULT	ADOLESCENT	YOUNG ADULT
NOVELTY-REACTIVITY (10 min in environment)	**CPP**	No CPP	No CPP	No CPP
	No CPP	No CPP	No CPP	**CPP**
NOVELTY-HABITUATION (1h in environment)	No CPP	No CPP	No CPP	**CPP**
	No CPP	**CPP**	No CPP	No CPP
Free choice				
NOVEL OBJECT RECOGNITION TASK	No CPP	No CPP	No CPP	No CPP
	No CPP	No CPP	No CPP	No CPP
NOVEL ENVIRONMENT TEST	No CPP	No CPP	No CPP	No CPP
	No CPP	**CPP**	No CPP	**CPP**
HOLE BOARD TEST	No CPP	No CPP	No CPP	**CPP**
	CPP	No CPP	**CPP**	No CPP

Level of novelty seeking **CPP** with cocaine
low (1 mg/kg)
high

entering the novel environment and the percentage of time spent in the novel environment are scored. Depending on their percentage of novelty-preference animals are categorized as high or low novelty seekers (HNS or LNS). In fact, the novel environment test is the most popular method used to classify animals according to their high or low exploratory behavior.

The Spontaneous Alternation Task

In this paradigm, animals are placed in the stem of a T- or Y-maze [65, 68] that permits entry into one arm (trial 1). In some cases, animals may choose the arm (free trial procedure) and in other cases they are forced to enter one of the arms (forced trial procedure). In both cases, animals are confined to the arm for 30 s. In the test phase (trial 2) the animals are free to choose the same or the alternative arm. It is important to note that rodents show a higher preference for the novel arm in the forced trial procedure than in the free trial procedure. This appears to be the case because rodents have a bias for one side of the apparatus and choose freely this side on both occasions [68–70].

The Novel Object Recognition Task

Initially, this task was developed for evaluating directed exploration in rats [71], and was later adapted for mice, with minor modifications [51]. Besides measuring novelty-seeking, this test also involves recognition memory, detection, and processing of the novel object [72].

Animals are free to explore one or several different objects (usually between 2 and 8) in an open field or playground maze on consecutive days (during 3, 5, or 10 min) [66, 67, 71, 73,

74]. Once they have become habituated to these objects, one of them is replaced by a new object and the approach behavior of the animal to the novel vs. familiar object is assessed [75]. Animals respond by preferentially exploring the substituted objects over the nonsubstituted objects [74]. Typically scored behaviors are the latency to make contact with the novel object, the percentage of time spent exploring novel and familiar objects, and the number of approaches/explorations of the novel object. Object exploration is defined as intentional contact of the animal's nose or front paws with the novel object.

In our laboratory we used a procedure similar to that described by Frick and Gresack [74]. We performed a single trial novel object recognition in an open arena (24 × 24 cm) illuminated by a dim light (22 W, 1080 lm) with two small river stones (1.5 cm wide × 3 cm high) and a small color toy made of nontoxic plastic. These objects were fixed to the floor with Velcro tape in opposite corners of the open field (5 cm from the walls). A camera recorder fixed to the ceiling allowed the arena to be visualized. Each mouse completed a daily trial (lasting 10 min) on 4 successive days. In the habituation phase (trial 1), the mouse was placed in the center of the empty open field box and allowed to explore it freely. In the sample phase (trials 2 and 3), two of the stones were placed in opposite corners of the box and the mouse was allowed to explore them. In the test phase (trial 4), one of the stones was replaced with the small colored toy in order to assess novel object exploration.

The Hole Board Test

This procedure is briefly explained here because it will be described at greater length in the following sections. The apparatus consists of a square box with equidistant holes in the floor. Sensors inside the holes detect the number of head dips performed by the animals. The latency to the first head dip and number of dips are used to classify animals as high or low novelty seekers [65, 76, 77]. Furthermore, this test can also be used to estimate the emotional response of animals when facing an unfamiliar environment and to assess anxiety state [77–81]. According to the propensity of an animal to explore the apparatus (measured by the number of head dips) it is classified as high novelty seeker (HNS) or low novelty seeker (LNS) [65, 76]. Although this procedure shares the inescapable condition with the novelty response in the open field, the hole board test is considered a useful tool to evaluate novelty-preference in rodents [78, 82], as they may perform dipping behavior. In fact, some authors have verified that the results observed in the hole board are independent of locomotor activity [2, 81, 83].

The Novel-Object Place Conditioning Paradigm

In this model, first described by Bevins and Bardo [84], the conditioned rewarding effects of novelty are evaluated. The place conditioning apparatus consists of two well differentiated compartments (by means of distinctive stimuli such as the color of the walls

or the texture of floor). During the Conditioning phase, animals are confined for 10–15 min to one compartment containing a new object (paired side) or to the other compartment, which is free of objects (unpaired side), for 5 or 8 consecutive days [84, 85]. On postconditioning (test) day, rodents are allowed to explore both compartments freely for 10 min without any object being present inside the apparatus. High novelty seekers spend more time in the novelty-paired compartment.

Object Preference on the Hole Board

This procedure combines the object preference and hole board tests. Two objects are placed in two separate holes of the hole board and the preference for novelty is calculated by the sum of the time engaged in head dipping in the holes that contain objects divided by the total time engaged in dipping [65].

1.4 Influence of Novelty-Seeking on Drug-Induced Reward

Empirical evidence indicates that the high novelty-seeking profile represents an increased risk of drug use in comparison to low novelty-seeking. However, novelty-seeking behavior does not always predict drug reward, probably due to variations in the procedure used to evaluate novelty-seeking (*see* Subheading 1.3) and/or the paradigms used to measure the rewarding effects of drugs of abuse. The most common animal models of reward are the drug self-administration and the conditioned place preference (CPP) paradigms.

Intravenous drug self-administration is a widely used animal model of human drug abuse and dependence, and allows the primary hedonic or reinforcing effects of drugs of abuse to be measured [86]. In a previous work we reviewed the main studies that have used this paradigm to evaluate the influence of the novelty-seeking phenotype on the reinforcing effects of psychostimulant drugs [87]. Most studies have used male adult rats and have evaluated the novelty-seeking trait by means of the locomotor response in an inescapable novel environment. Results obtained with this paradigm are controversial, since some studies have reported that HR rats self-administer more amphetamine and cocaine than LR rats [54, 57, 58, 62, 88–94] but not in others [64, 67, 90, 95–97]. Furthermore, HR and LR rats do not differ regarding the self-administration of methylphenidate [98] and MDMA [99]. Similar discrepant results have been obtained using free-choice novelty-seeking paradigms with HNS male adult rats self-administering more amphetamine, cocaine, and methylphenidate than their LNS counterparts in some studies [64, 67, 95, 97, 98], but not in others [62, 67]. In male adult mice, the novelty-seeking trait facilitates the self-administration of cocaine in inescapable and free-choice paradigms [100]. The influence of novelty-seeking on the reinforcing effects of alcohol has only been studied in two works, which also rendered divergent results. Bienkowski et al. [101] found that response to novelty in the open field and

novel object test did not predict individual differences to oral operant ethanol self-administration. Conversely, Nadal et al. [102] reported a positive relationship between the level of activity in a novel environment and the acquisition of alcohol self-administration, although only under a FR3 schedule. No research to date has evaluated the effects of novelty-seeking on cannabinoid self-administration.

The conditioned place preference (CPP) induced by drugs of abuse has become a popular alternative to the drug self-administration paradigm for evaluating the sensitivity of individuals to the rewarding properties of these substances [103–106]. In the CPP paradigm, the incentive properties of a drug are assessed in drug-free animals according to the amount of time they spend in an environment previously paired with the drug's effects (see details of the procedure in Subheading 2.2.3). In a previous work we reviewed the main studies that have used this paradigm to evaluate the influence of the novelty-seeking phenotype on the reinforcing effects of psychostimulant drugs [87]. When assessing the locomotor response to novelty in an inescapable novelty-seeking paradigm, no differences were found between HR and LR rats in the CPP induced by cocaine or amphetamine [107–112], while HR rats were less sensitive to amphetamine CPP [113]. Similar negative results with respect to cocaine CPP (LR > HR, LR = HR) have been observed in mice [114–116], except in the case of young adult mice exposed to the new environment for only 10 min [114]. Conversely, most studies using free-choice novelty-seeking paradigms to classify animals have reported that HNS rats and mice acquired amphetamine and cocaine CPP more readily than LNS animals [66, 73, 111, 117]. Preference for novelty has also been related to a higher sensitivity to the rewarding effects of morphine [118–120] and nicotine [76], but not of alcohol [83].

Individual characteristics of experimental animals (species, strain, sex and age) and the paradigm of novelty-seeking used to categorize the animals as high or low novelty seekers are essential variables that can affect the results observed. As can be seen in Table 2, the differential sensitivity of HNS and LNS mice to the rewarding effects of 1 mg/kg of cocaine in the CPP paradigm has only been detected with some paradigms of novelty-seeking in function of the sex and age of mice. When we tested adolescents of both sexes, the higher sensitivity of HNS to cocaine CPP was detected only in the hole board paradigm. Similarly, when we evaluated adults of both sexes, the novel environment test was found to be useful to discriminate the higher sensitivity of HNS mice. Finally, the HR profile in an inescapable novel environment is also predictive of enhanced vulnerability to developing cocaine CPP in adult female mice.

1.5 Novelty-Seeking and Adolescent Drug Exposure

In a series of studies from our laboratory, we have evaluated how the novelty-seeking phenotype modulates the long-lasting effects of adolescent exposure to psychostimulant drugs, alcohol or cannabinoids on the subsequent rewarding properties of drugs of abuse in the CPP paradigm (*see* Table 3). Pretreatment with drugs of abuse during adolescence also induces other behavioral and biochemical effects that in some cases are influenced by the novelty-seeking trait (*see* Tables 4, 5, and 6).

1.5.1 Cocaine

In several studies performed in our laboratory we have observed that rodents that are more responsive to novelty (HR and HNS) develop CPP with a subthreshold dose of cocaine, which is ineffective in inducing CPP in low novelty seeker (LNS) and naïve mice [66, 114]. Moreover, we have seen that the level of novelty-seeking also influences the effects of cocaine exposure during adolescence on the subsequent vulnerability of mice to cocaine and MDMA [77] (*see* Table 3). Adolescent HNS mice exposed to cocaine binges are more sensitive to the conditioned rewarding effects of low doses of cocaine and MDMA in adulthood. Age is a critical factor in these effects, since exposure to the same schedule of cocaine binges in adult mice do not induce this subsequent enhanced vulnerability to the rewarding effects of cocaine and MDMA, irrespective of the novelty-seeking profile. Adolescent cocaine binges also produce subtle, long-term changes in the behavior of mice, particularly in those with an HNS profile, such as a decrease in exploratory behavior, increased locomotor reactivity and greater impulsivity-like behaviors [77] (*see* Tables 4, 5, and 6). These alterations could be associated with the increased vulnerability of HNS cocaine-treated mice to develop cocaine and MDMA CPP in adulthood.

1.5.2 MDMA

As commented on before, the novelty-seeking profile is not significantly correlated with either acquisition of MDMA self-administration or drug-seeking in rats [99]. However, we have observed that pretreatment with binges of cocaine during adolescence induces an increase in the conditioned rewarding effects of MDMA only in HNS mice [77] (*see* Table 3). Similarly, we have detected a higher sensitivity of HNS to the conditioned rewarding effects of low doses of cocaine and MDMA in mice exposed to binges of MDMA during adolescence [122] (*see* Table 3). In addition, only HNS mice show other behavioral consequences of adolescent exposure to MDMA binges, such as an increase in social and aggressive behaviors. HNS MDMA-treated mice engage in more social contacts, but also behave more aggressively, than LNS mice [122] (*see* Table 4). On the other hand, a long-lasting anxiolytic effect after exposure to a high dose of MDMA (20 mg/kg) was observed only in LNS mice (they spent more time in the open arms of the EPM than HNS mice). Thus, it can be deduced that LNS mice display less emotional reactivity than their HNS counterparts

Table 3
Mouse studies of the influence of novelty-seeking profile in the long-term effects of adolescent drug exposure on later sensitivity to drug reward (CPP)

		Age hole board	Pretreatment			CPP		Dose mg/kg			
Animal	Sex	PND	PND	Drug	Dose	PND	Drug		Sensitivity to drug reward	Reference	Conclusion
OF1 mice	Male	Adolescent, 31	33–34, 37–38, 41–42, 45–46	Saline		67–74	Cocaine	1	HNS = LNS, no CPP	[121]	Adolescent HNS exposed to alcohol are more vulnerable to reinstatement of cocaine CPP
				Alcohol binge	2.5 g/kg	67–74	Cocaine	1	HNS = LNS, CPP; reinstatement only in HNS		
					2.5 g/kg	67–74	Cocaine	6	HNS = LNS, CPP; reinstatement only in HNS		
					0 g/kg	67–78	MDMA	1.25	HNS = LNS, no CPP		
					2.5 g/kg	67–78	MDMA	1.25	HNS = LNS, CPP		
					2.5 g/kg	67–78	MDMA	2.5	HNS = LNS, CPP		
OF1 mice	Male	Adolescent, 33	34–35, 36–38, 42–45	Saline		67–74	Cocaine	1	HNS = LNS, no CPP	[77]	Adolescent HNS exposed to cocaine are more sensitive to the rewarding effects of cocaine and MDMA CPP in adulthood. Cocaine exposure during adulthood do not increase the rewarding effects of cocaine
						67–78	MDMA	1.25	HNS = LNS, no CPP		
				Cocaine binge	5–10–15 mg/kg	67–74	Cocaine	1	CPP only in HNS		
						67–78	MDMA	1.25	CPP only in HNS		
		Young adults, 60	61–62, 63–65, 68–72	Saline		93–100	Cocaine	1	HNS = LNS, no CPP		
						93–104	MDMA	1.25	HNS = LNS, no CPP		
				Cocaine binge	5–10–15 mg/kg	93–100	Cocaine	1	HNS = LNS, no CPP		
						93–104	MDMA	1.25	HNS = LNS, no CPP		
OF1 mice	Male	Adolescent, 28	33, 34, 40, 41	Saline		67–74	Cocaine	1	HNS = LNS, no CPP	[122]	Adolescent HNS exposed to MDMA are more sensitive to the rewarding effects of cocaine and MDMA in adulthood
						67–78	MDMA	1.25	HNS = LNS, no CPP		
				MDMA binge	10 mg/kg	67–74	Cocaine	1	CPP only in HNS		
						67–78	MDMA	1.25	CPP only in HNS; reinstatement only in HNS		

(continued)

Table 3
(continued)

Animal	Sex	Age hole board PND	Pretreatment PND	Drug	Dose	CPP PND	Drug	Dose mg/kg	Sensitivity to drug reward	Reference	Conclusion
OF1 mice	Male	Adolescent, 26	26–30			30–41	WIN	0.05	HNS = LNS, no CPP	[123]	Adolescent HNS are more sensitive to the rewarding effects of WIN 55212-2. Adolescent HNS exposed to this cannabinoid agonist are more vulnerable to the rewarding effects of cocaine (longer CPP and reinstatement)
						30–41	WIN	0.075	CPP only in HNS		
				VEHICLE	0 mg/kg	34–45	WIN	0.05	HNS = LNS, no CPP		
				WIN 55212-2	0.1 mg/kg	34–45	WIN	0.05	HNS = LNS, no CPP		
				SR 141716A	1 mg/kg	34–45	WIN	0.05	HNS = LNS, no CPP		
				VEHICLE	0 mg/kg	34–41	Cocaine	1	HNS = LNS, no CPP		
				WIN 55212-2	0.1 mg/kg	34–41	Cocaine	1	HNS = LNS, CPP; reinstatement only in HNS		
				SR 141716A	1 mg/kg	34–41	Cocaine	1	HNS = LNS, no CPP		
				WIN 55212-2	0.1 mg/kg	34–41	Cocaine	6	HNS = LNS, CPP and reinstatement CPP duration: HNS > LNS		

Table 4
Mouse studies about the influence of the novelty-seeking profile in the long-term effects of adolescent drug exposure on later social behavior

Animal	Sex	Age hole board PND	Pretreatment PND	Drug Binge	Dose	Social interaction test	Reference
OF1 mice	Male	Adolescent, 31	33–34, 37–38, 41–42, 45–46	Alcohol	0 g/kg 1.25 g/kg 2.5 g/kg	PND 71, HNS = LNS PND 71, HNS = LNS PND 71, HNS = LNS (HNS > HNS saline in non-social exploration) (HNS < HNS saline in threat and attack)	[121]
OF1 mice	Male	Adolescent, 33	34–35, 36–38, 42–45	Cocaine	0, 0, 0 mg/kg 5–15–25 mg/kg	PND 82–84, HNS = LNS PND 82–84, HNS = LNS	[77]
OF1 mice	Male	Adolescent, 28	33, 34, 40, 41	MDMA	0 mg/kg 10 mg/kg 20 mg/kg	PND 75, HNS = LNS PND 75, increased social investigation, threat and attack only in HNS HNS > LNS in social investigation, threat and attack PND 75, increased attack only in HNS HNS > LNS in attack	[122]

Table 5
Mouse studies about the influence of the novelty-seeking profile in the long-term effects of adolescent drug exposure on later behavior in the elevated plus maze

Animal	Sex	Age Hole board PND	Pretreatment PND	Drug Binge	Dose	Elevated plus maze (EPM)	Reference
OF1 mice	Male	Adolescent, 31	33–34, 37–38, 41–42, 45–46	Alcohol	0 g/kg 1.25 g/kg 2.5 g/kg	PND 67, HNS = LNS PND 67, HNS = LNS (time in OA, %time OA: HNS > HNS saline) PND 67, HNS = LNS (time in OA, %time OA, HNS > HNS saline) (entries OA, HNS > HNS saline, LNS > LNS saline)	[121]
OF1 mice	Male	Adolescent, 33	34–35, 36–38, 42–45	Cocaine	0, 0, 0 mg/kg 5–15–25 mg/kg	PND 67, HNS = LNS PND 67, HNS = LNS, cocaine pre-treatment reduced latency to enter OA only in LNS	[77]
OF1 mice	Male	Adolescent, 28	33, 34, 40, 41	MDMA	0 mg/kg 10 mg/kg 20 mg/kg	PND 64, HNS = LNS PND 64, increased time and %time in OA only in LNS PND 64, increased time and %time in OA only in LNS time and % time in OA, HNS < LNS	[122]

Table 6
Mouse studies about the influence of the novelty-seeking profile in the long-term effects of adolescent drug exposure on later behavioral and neurochemical measures

Animal	Sex	Age	Pretreatment PND	Drug Binge	Dose	Other measures	Reference
OF1 mice	Male	Adolescent, 31	33–34, 37–38, 41–42, 45–46	Alcohol	0 g/kg 1.25 g/kg 2.5 g/kg	Novel object. PND 70, HNS = LNS Novel object. PND 70, HNS = LNS (%time exploring novel object, LNS < LNS saline) Novel object. PND 70, latency to explore novel object: HNS < LNS (%time exploring novel object: LNS < LNS saline) (number of explorations: LNS < LNS saline) (latency to explore novel object: LNS > LNS saline) (number of explorations: HNS < HNS saline)	[121]
OF1 mice	Male	Adolescent, 33	34–35, 36–38, 42–45	Cocaine	0, 0, 0 mg/kg 5–15–25 mg/kg	Actimeter. PND 68–69, HNS = LNS PND 68–69, HNS = LNS, cocaine pretreatment increased activity in both groups	[77]
OF1 mice	Male	Adolescent, 28	33, 34, 40, 41	MDMA	0 mg/kg 10 mg/kg 20 mg/kg	Biogenic amines. PND 76, HNS < LNS striatal DOPAC PND 76, increases striatal DA and DA turnover only in LNS PND 76, HNS < LNS striatal DOPAC and DA turnover reduces striatal DA and increases DA turnover in LNS HNS < LNS striatal DA turnover	[122]

[122] (*see* Table 5). The long-term effect of MDMA binges on striatal DA is also modulated by the novelty-seeking trait, as MDMA exposure induces a significant decrease of striatal DA and an enhanced DA turnover in this structure only in LNS mice. Furthermore, irrespective of the MDMA treatment, we found that LNS exhibited lower levels of DOPAC, which may be related with the behavioral effects observed [122] (*see* Table 6). The changes in the behaviors evaluated in the study suggest that experience of MDMA during adolescence alters the way in which subjects interact with their environment in adulthood; thus, certain individuals will be more prone to suffering long-lasting effects. In particular, HNS become more sensitive to the rewarding effects of cocaine and MDMA; furthermore, they engage in more social interaction but are more aggressive, which could also increase drug use.

1.5.3 Alcohol

Several studies have revealed a relationship between ethanol use and the novelty-seeking trait. High sensation seekers show increased alcohol intake and experience more positive effects after its consumption [46]. Furthermore, the novelty-seeking trait predisposes individuals to develop alcohol-related problems, including alcoholism and enhanced vulnerability to relapse after periods of detoxification [124, 125].

In experimental animals, adolescent exposure to chronic alcohol increases the tendency of animals to engage in more exploratory or novelty-seeking behaviors [22], and exposure to alcohol binges during adolescence induces long-lasting behavioral consequences that are influenced by the novelty-seeking phenotype [121]. As shown in Table 3, although adolescent male mice exposed to a binge pattern of EtOH develop CPP with subthreshold doses of cocaine and MDMA regardless of their novelty-seeking profile, HNS animals have been found to require more extinction sessions for cocaine CPP to be extinguished than their LNS counterparts (8 sessions vs. 4 sessions). Furthermore, after extinction is achieved, only HNS mice show reinstatement of cocaine CPP by a priming dose of this drug [121]. Extinction measures the motivational properties of drugs, which are reflected by the persistence of drug-seeking behavior in the absence of the drug, while reinstatement of the extinguished preference is a reliable model of the craving and relapse that characterize drug addiction [106]. Thus, these results suggest that the HNS trait enhances motivation for cocaine and vulnerability to develop addiction in mice exposed to alcohol during adolescence.

In contrast with that observed with cocaine, the acquisition, extinction, and reinstatement of the CPP induced by MDMA is not affected by the novelty-seeking profile of mice exposed to alcohol during adolescence (*see* Table 3). Such results have been observed

by Bird and Schenk [99], who reported that the level of novelty-seeking does not significantly correlate with MDMA self-administration in rats. The differential modulation by the novelty-seeking phenotype of the effects of adolescent EtOH exposure on cocaine and MDMA reward might be due to the unique pharmacology of MDMA, which, unlike other psychostimulant drugs, preferentially enhances synaptic 5-HT [126]. In fact, although serotonin mediates the response to novelty-seeking [127, 128], DA plays a more important role [23].

Other long-term effects of exposure to EtOH during adolescence are modulated by the novelty-seeking trait [121]. In particular, EtOH pretreatment is known to exert an anxiolytic effect only in HNS mice (*see* Table 4). Similarly, adolescent exposure to EtOH induces changes in aggressive behavior in HNS mice only, for instance, an increase in nonsocial exploration and a reduction in threat and attack (*see* Table 5). Finally, in a novel object recognition task, adolescent alcohol exposure was shown to increase the number of explorations of the novel object only in HNS; conversely, in LNS mice, alcohol pretreatment reduced the number of explorations and the time spent exploring the novel object, while it increased the latency to explore it (*see* Table 6).

1.5.4 Cannabinoids

Consumption of cannabis, the most used illegal drug, usually begins during adolescence.

Recently, the problematic use of cannabis has increased in adolescent individuals, a fact that can induce negative consequences, including enhanced vulnerability to develop dependence and/or higher propensity to later consumption of other drugs [129, 130] such as cocaine [131–133]. In humans, cannabinoid pre-exposure increases the severity of cocaine withdrawal symptoms and relapse to cocaine dependence [134]. Similarly, in adolescent rodents, exposure to cannabinoid agonists increases self-administration [135–140] and modifies the acquisition and reinstatement of the CPP induced by different drugs of abuse, such as morphine [141], MDMA [142] and cocaine [123].

The novelty-seeking phenotype influences the sensitivity of mice to the conditioned rewarding effects of the cannabinoid agonist WIN 55212-2. In particular, we have observed that HNS mice acquire CPP after conditioning with 0.075 mg/kg, a dose that is ineffective in inducing conditioned reward in LNS [123]. In the study in question, we also observed the influence of the novelty-seeking trait on the effects of adolescent cannabinoid exposure (*see* Table 3). In HNS and LNS pre-exposed to the CB1 agonist WIN 55212-2 no differences were observed between the two in the subsequent CPP induced by this drug. Furthermore, pretreatment with WIN 55212-2 increases the rewarding effects of a low dose of cocaine (1 mg/kg), irrespective of the novelty-seeking profile of the

mice. However, it is important to note that HNS mice are more resistant to the extinction of CPP and more sensitive to reinstatement of CPP after extinction. In particular, HNS mice require twice as many extinction sessions as LNS mice to achieve extinction of a cocaine CPP. Furthermore, priming with 0.5 mg/kg and 3 mg/kg reinstates the CPP induced by 1 mg/kg and 6 mg/kg of cocaine, respectively, in HNS mice only. On the other hand, pretreatment with the cannabinoid antagonist rimonabant does not increase the sensitivity of mice to the conditioned rewarding effects of WIN 55212-2 or cocaine [123]. These results support the idea that not all subjects are equally vulnerable to the sensitization of the brain reward system induced by stimulation of the cannabinoid system during adolescence, but that those with an HNS profile will be particularly affected.

2 Evaluating How Behavior in the Hole Board Test Modulates the Effects of Adolescent Drug Exposure on Subsequent Drug-Induced CPP

2.1 Materials

2.1.1 Subjects

Strain

With the exception of the studies discussed in the last section of the chapter, we always use mice of the OF1 strain.

The **Age** of animals is a relevant factor (*see* **Notes 1** and **2**); for example, novelty-seeking behavior is typically more pronounced in adolescent than in adult rodents (*see* **Note 1**). In addition, adolescent rodents show enhanced sensitivity to the rewarding properties of novelty [85] and psychostimulant drugs [28, 143–149], which is reflected by an enhanced vulnerability to developing CPP [39, 145].

Adolescent mice also experience less aversive effects of addictive drugs and reduced withdrawal symptoms (*see* [39]), and this is important to bear in mind when selecting the drug dose to evaluate the effects of novelty-seeking in drug-induced CPP (*see* **Note 2**).

To evaluate the novelty-seeking profile and to model adolescent drug exposure, we usually choose to employ mice of PND 26–45, which is considered a conservative age range during which animals of both genders and most breeds are expected to exhibit adolescent-typical neurobehavioral characteristics [36, 150].

Sex is another factor that must be considered. Although the estrous cycle does not affect the locomotor response to novelty-induced behavioral tests [58, 151], sex differences have been reported in the novel object recognition task [74, 151] (*see* Table 1). Furthermore, it is known that women show a faster onset of addiction and require treatment sooner than men [152–155]. Sex differences in the rewarding effects of drugs of abuse have also been observed in rodents [152, 154–156] (*see* **Note 3**).

To study the influence of the novelty-seeking profile on vulnerability to cocaine CPP we have used early–middle adolescent (PND

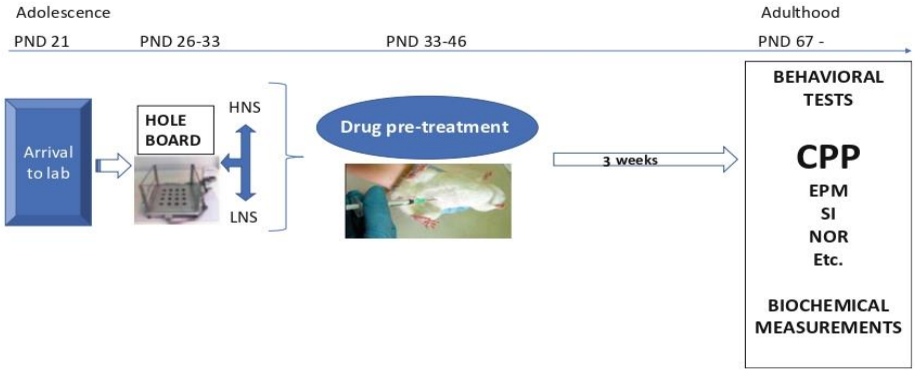

Fig. 2 Timeline of studies about the influence of novelty-seeking profile in the long-term effects of adolescent drug exposure on behavioral tests (*CPP* conditioned place preference, *EPM* elevated plus maze, *SI* social interaction, *NOR* novel object recognition) and biochemical measures

28–35) and late adolescent (PND 49–56) mice of both sexes of the OF1 outbred strain [66, 114]. As can be seen in Table 2 the results obtained are in function of the paradigm of novelty-seeking used.

However, to evaluate the consequences of previous drug exposure for the subsequent rewarding effects of drugs of abuse according to novelty-seeking profile, we typically use male adolescent mice [77, 121–123]. In brief, the experimental steps are as follows (*see* Fig. 2 and the detailed schedules in Tables 3, 4, 5, and 6):

1. Mice are acquired commercially from the supplier Charles River (France).
2. Mice arrive at the laboratory on PND 21.
3. The hole board test is performed between PND 26 and PND 33 (early adolescence).
4. Typically, mice receive the drug treatment between PND 33 and PND 46 (middle adolescence). However, in the case of cannabinoids adolescent treatment is administered during early adolescence (PND 26–30).
5. Behavioral tests and biochemical measurements are performed in adulthood, usually from PND 67.

Conditions in the Laboratory

When the animals arrive at the laboratory, they are housed in groups of four in plastic cages (28 × 28 × 14.5 cm) for 5–10 days prior to initiating experimental procedures. The conditions of the laboratory are as follows:

- Constant temperature (21 ± 2 °C),
- Relative humidity of 60%,

- Reversed light schedule (white lights on: 19.30–07.30 h),
- Food and water available ad libitum (except during the behavioral test).

Procedures involving mice and their care are always conducted in conformity with national, regional and local laws and regulations, which are in accordance with the European Communities Council Directives (2010/63/EU). The protocols are always approved the University of Valencia's Ethical Committee on Animal Experimentation.

Animals are handled briefly on the 2 days preceding initiation of the experimental procedures in order to reduce their stress levels (*see* **Note 4**).

2.1.2 Drugs of Abuse

Alcohol: *According to the National Institute on Alcohol Abuse and Alcoholism, an alcohol binge can be defined as a pattern of alcohol consumption that results in a blood alcohol concentration of 0.08-g% or higher, that is, the consumption of five (four in the case of females) or more drinks in the space of about 2 h. To imitate this pattern of binge consumption, we administer twice-daily injections (separated by a 4-h interval) of 1.25 or 2.5 g/kg ethanol (Merck, Madrid, Spain) several times for a week (for more details see Subheading 2.2.2). The control group is injected with repeated injections of physiological saline (NaCl 0.9%), also used to dissolve the ethanol, following the same schedule as the binge pattern.*

Cocaine: *(Laboratorios Alcaliber S.A., Madrid, Spain) is usually administered to induce CPP or as pretreatment during adolescence. This drug is diluted in physiological saline (0.1 mg/mL), which is administered to controls. The cocaine dose most used for CPP (1 mg/kg) is based on previous studies [157] in which it was shown to be a subthreshold dose, that is, ineffective in inducing CPP in naïve mice [66, 157]* (see **Note 5**). *When administered during adolescence in order to evaluate its long-term effects, mice are frequently treated with cocaine binges. We usually employ one of two schemes of increasing doses from 5 to 10 mg/kg or from 5 to 25 mg/kg (for more details see Subheading 2.2.2).*

MDMA: *(3,4-methylenedioxy-metamphetamine hydrochloride; racemic mixture) is commercially acquired from Laboratorios Sigma-Aldrich (Spain) or provided by the Agencia Española del Medicamento, Ministerio de Sanidad, Política Social e Igualdad, Madrid, Spain. For CPP experiments we use doses of 1 or 2.5 mg/kg of MDMA dissolved in physiological saline, which are ineffective in naïve mice [158]. For pretreatment binges, we use 10 or 20 mg/kg of MDMA (for more details see Subheading 2.2.2).*

WIN 55212-2: *is a cannabinoid CB1 agonist used to study the effects of cannabinoid drugs such as cannabis. WIN 55212-2 is commercially acquired from Tocris, Biogen Cientifica, S.L. (Madrid,*

*Spain) and dissolved with saline and Tween 80 (Sigma-Aldrich, Madrid, Spain) at 0.01% (0.01 mL of Tween dissolved in 100 mL of saline) (*see **Note 6**). *The doses administered in the CPP paradigm (0.05, 0.075 mg/kg) are selected on the basis of previous studies which have confirmed the subthreshold dose that does not induce CPP in naïve mice [141, 159]. For adolescent pretreatment, a higher dose of WIN 55212-2 (0.1 mg/kg) is used; mice receive one injection per day for 5 days (see more details in Subheading 2.2.2). Such cannabinoid exposure during adolescence is effective in increasing the CPP induced by MDMA in adulthood [142]. To evaluate the involvement of CB1 receptors in the effects of WIN 55212-2 (or other drugs of abuse), we use* **SR 141716A** *(rimonabant), commercially acquired from Sanofi Recherche (Montpellier, France), administered at a dose of 1 mg/kg, since this dose blocks CB1 receptors and does not act as an inverse agonist of these receptors [160].*

All injections are administered intraperitoneally, at a constant volume of 10 mL/kg (0.01 mL/g) (*see* **Note 7**).

2.1.3 Apparatus

Hole Board

The hole board consists of a box (28 × 28 × 20.5 cm) with walls made of clear Plexiglas. In the floor of the box there are 16 equidistant holes with a diameter of 2.3 cm (*see* Fig. 2). Photocells below the surface of the holes detect the number of times the mouse performs a head dip (Med Associates, CIBERTEC, SA, Spain). A computerized system records the number of times a mouse explores a specific hole and the total frequency of dips (Activity Monitor v.7) (*see* Figs. 3 and 4).

Conditioned Place Preference Boxes

The apparatus consists of eight identical Plexiglas place conditioning boxes comprised of two equally sized compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm). The compartments have different colored walls (black vs. white) and distinct floor textures (smooth in the black compartment and rough in the white one). Four infrared light beams in each compartment of the box and six in the central area allow the position of the animal and its crossings from one compartment to the other to be recorded (*see* Fig. 4). The equipment is controlled by an IBM PC computer using MONPRE 2Z software (CIBERTEC, SA, Spain) (*see* Fig. 4).

2.2 Methods

2.2.1 Hole Board

As commented on before, the hole board test evaluates the tendency of rodents to explore a new environment in a free-choice procedure. This test was developed in 1962–1964 by Boissier and Simon [78, 82], and is a simple and useful procedure to assess the response of an animal to an unfamiliar setting [81]. The exploratory behavior measured in this test is the number of head dips, which represents exploratory tendencies distinct from general locomotor

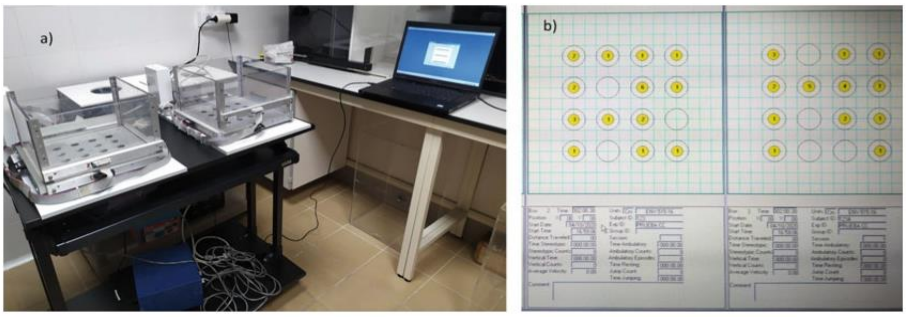

Fig. 3 The hole board test. (**a**) Two hole board apparatuses connected to the interphase and the computer that controls the apparatus. (**b**) Register of the mouse's behavior (dips, distance traveled, velocity, etc.) on the computer screen. Holes visited are marked in yellow; the number inside the hole indicates the number of dips in the hole performed by the mouse

Fig. 4 Experimental room for place conditioning. Each computer controls four CPP boxes. Each box has two different compartments (black and white) separated by a small central gray corridor (see more details in the text). A table in the middle of the room is used to administer injections immediately before placing the mouse in the corresponding compartment

activity. Number of head dips is a useful measure to study the relationship between novelty-seeking and drug abuse [83].

Adolescent animals perform the hole board test after a 5–10-day period of acclimatization to the laboratory, during the dark phase (9.00–12.00 h). The illumination in the experimental room is provided by four neon tubes fixed to the ceiling (light intensity of 110 lux at 50 cm above floor level) (*see* **Note 8**).

At the beginning of the test, the mouse is placed in one corner of the hole board and allowed to explore it freely for 10 min. The total number of heap dips and the latency to perform the first head dip is scored (*see* **Note 9**). The box is carefully cleaned with 70% alcohol at the end of each test.

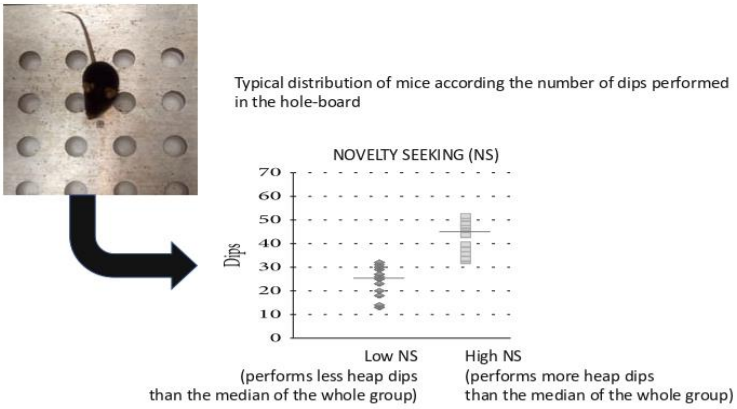

Fig. 5 Classification of mice as HNS and LNS according to the number of dips performed in the hole board

We use a median-split analysis to study the effects of novelty-seeking on the behavioral effects of different drugs of abuse [77, 114, 121–123]. Mice are defined as high novelty seekers (HNS) or low novelty seekers (LNS) according to whether the number of head dips they perform is higher or lower than the median of the group (HNS or LNS, respectively). We use the median to classify mice as HNS versus LNS, because it is a standard measure when a set of data has been arranged in order of magnitude and is less affected by outliers (*see* Fig. 5). As shown in Table 2, the hole board is the best test to predict which adolescent male mice will be more sensitive to the conditioned rewarding effects of cocaine in the adulthood (*see* **Note 10**). After the mice have been classified as HNS or LNS, they are randomly assigned to a drug treatment, ensuring that in each group of treatment approximately half the mice are HNS and the other half are LNS (*see* Fig. 5).

2.2.2 Pharmacological Treatment

After the adolescent mice have been classified as HNS or LNS (according to the number of dips performed in the hole board), both groups are treated with a drug of abuse, usually between PND 28–42, considered a conservative age range during which mice are expected to exhibit neurobehavioral characteristics typical of adolescence [150].

Cocaine Binges

To evaluate how the novelty-seeking trait influences the effects of adolescent cocaine exposure (PND 34–45) and the subsequent rewarding properties of cocaine and MDMA, a pretreatment of three injections per day is administered at 1-h intervals, with the dose being increased at different points of this 12-day period. From PND 34–35, mice receive 5 mg/kg; from PND 36 to PND

38, they receive 10 mg/kg; and from PND 41–45, they receive 15 mg/kg. For comparison, the same schedule of administration of cocaine can be administered to adult mice (PND 61 and 72). To evaluate how such adolescent cocaine binges alter behavior in the elevated plus maze (anxiety) and social interaction test (agonistic encounter with a cospecific mouse), the same schedule of cocaine administration is applied, but with higher doses (5, 15 and 25 mg/kg) (*see* **Note 11**). Physiological saline injections following the same schedule as that used for cocaine are administered to control groups. The long-term consequences of these binges on different behavioral tests are studied 3 weeks after the last drug administration (*see* Fig. 2).

MDMA Binges

Mice receive eight injections of MDMA (at doses of 0, 10, or 20 mg/kg) over a 2-week period according to the following schedule: twice-daily administrations (with a 4-h interval: at 9 am and 1 pm) on 2 consecutive days each week. In this way, mice are injected on PND 33, 34, 40, and 41. Behavioral tests are performed 3 weeks after exposure had finalized, when mice have entered adulthood. We use doses of 10 and 20 mg/kg to evaluate the long-term effects of binges on social behavior, anxiety and biochemical measures (dopamine, serotonin, metabolites and turnover), while only the dose of 10 mg/kg is used to evaluate the long-term effects of MDMA binges on drug-induced CPP.

Ethanol Binges

Mice are treated with EtOH (1.25 or 2.5 g/kg) or physiological saline on 2 consecutive days, at 48-h intervals, over a 14-day period to simulate a binge pattern such as that engaged in by human adolescents and young adults [161]. Each mouse receives 16 injections, according to the following schedule: twice daily injections (with a 4-h interval) on 2 consecutive days separated by an interval of 2 days during which no injections were administered. Animals are injected on PND 33, 34, 37, 38, 41, 42, 45, and 46. The control group receives the same schedule of injections, but of physiological saline. The long-term consequences of these binges on anxiety levels (elevated plus maze and social interaction test) and spontaneous motor activity are studied 3 weeks after the last administration of EtOH. To evaluate the consequences of adolescent ethanol binges on the subsequent rewarding effects of cocaine and MDMA we administer binges of 2.5 g/kg.

Pretreatment with Cannabinoid Drugs

Animals receive a daily injection of their respective treatment (vehicle, 0.1 mg/kg of WIN 55212-2, or 1 mg/kg of rimonabant) for 5 days (PND 26–30) and, after an interval of 3 days without any treatment, the CPP procedure is initiated [123].

2.2.3 Conditioned Place Preference

The CPP paradigm evaluates the positive and pleasant properties of stimuli (including the rewarding effects of addictive drugs) [103–106] in a rapid and simple way [162]. In this paradigm, contextual or environmental stimuli acquire secondary appetitive properties (conditioned rewarding effects) when paired with a primary reinforcer [104, 105]. Conditioned reward implies that animals attribute positive incentive value to the cues associated with the primary reinforcer (the drug of abuse), and thus perform free or voluntary responses to obtain access to said cues [163]. Under appropriate conditions, CPP can be sensitive to a wide range of substances, including opiates, psychostimulants, alcohol, and cannabinoids [162].

The procedure, unbiased in terms of initial spontaneous preference, is performed as described previously [164]. In the first phase, referred to as preconditioning (Pre-C), mice are allowed access to both compartments of the apparatus for 15 min (900 s) per day on 2 consecutive days. On Day 3, the time spent in each compartment over a 900-s period is recorded during the dark phase (between 10:00 and 14:00 h). We use a counterbalanced design to assign the mice of each group to the drug- and vehicle-paired compartment. After assigning the compartments, we perform an analysis of variance (ANOVA) with the data of the time spent in each compartment during the preconditioning phase. There must not be significant differences between the time spent in the compartment paired with the drug and that spent in the compartment paired with vehicle. This is an important requirement of the experimental procedure that avoids any preference bias prior to conditioning (*see* **Note 12**).

The second phase (conditioning) lasts 4 or 8 days, in function of the drug of abuse used to induce place conditioning. In the CPP induced by cocaine, the mice undergo 2 pairings per day, on 4 consecutive days. Animals receive an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they receive an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. The order of injections (cocaine or saline) is alternated every day. In the CPP induced by MDMA and the CB1 agonist, mice undergo only one pairing per day: animals conditioned with MDMA receive an injection of MDMA immediately before confinement in the drug-paired compartment for 30 min on Days 4, 6, 8, and 10, and receive physiological saline before confinement in the vehicle-paired compartment for 30 min on Days 5, 7, 9, and 11. Confinement is carried out in both cases by closing the guillotine door that separates the two compartments. The central area of the apparatus is never used during conditioning.

During the third phase, known as postconditioning (Post-C), the guillotine door separating the two compartments is removed (Day 8 in the case of cocaine, and Day 12 in the case of MDMA and

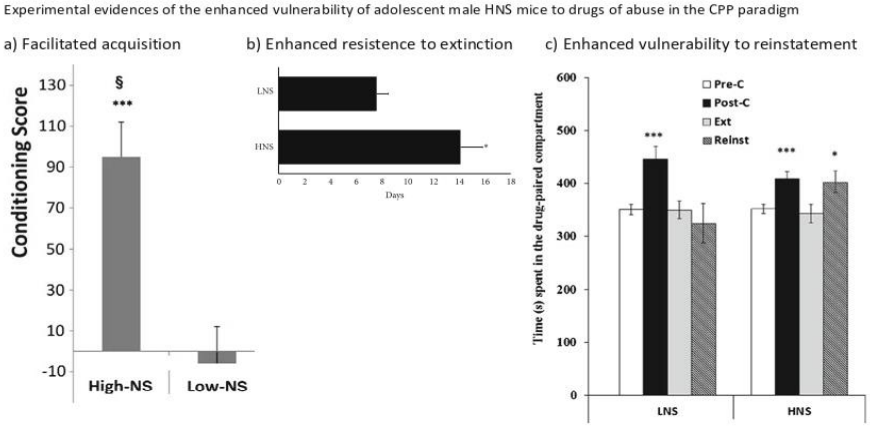

Fig. 6 Experimental evidence of the enhanced vulnerability of adolescent male HNS mice to drugs of abuse in the CPP paradigm. (**a**) Facilitated acquisition. (**b**) Enhanced resistance to extinction. (**c**) Enhanced vulnerability to reinstatement

WIN 55212-2), and the time spent by the untreated mice in each compartment during a 900-s observation period is recorded. Post-C tests are performed in the dark phase between 10:00 and 14:00 h.

The difference in seconds between the time spent in the drug-paired compartment during the Post-C test versus the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has developed.

The rewarding effects of drugs of abuse are evaluated 3 weeks after the end of adolescent drug pretreatments. Usually, subthreshold doses of drugs are used in order to evaluate the sensitivity of HNS and LNS mice to the conditioned reinforcing effects of these drugs (*see* **Note 5**); we generally use cocaine (1 mg/kg), MDMA (1.25 mg/kg), and WIN 55212-2 (0.05 and 0.075 mg/kg). The doses selected to induce CPP are based on previous studies [142, 157–159]. As can be seen in Fig. 6 and Table 3 there is a body of experimental evidence for the enhanced vulnerability of adolescent male HNS mice to drugs of abuse in the CPP paradigm. HNS mice acquire drug-induced CPP more readily (Fig. 6a), and pretreatment with psychostimulant drugs during adolescence enhances the CPP induced by these drugs of abuse only in HNS mice (*see* Table 3).

In some experiments, the groups that acquire CPP undergo extinction sessions to evaluate the persistence of CPP and the

subsequent vulnerability to drug-induced reinstatement of CPP. During each extinction session, the mouse is placed in the apparatus, without the guillotine doors separating the compartments, for 15 min. The extinction session is repeated every 72 h, until the time spent in the drug-paired compartment by each group is similar to that of Pre-C and differs from that of Post-C (Student's *t*-tests). In this way, all the animals in each group undergo the same number of extinction sessions, independently of their individual scores, as the criterion for extinction is a lack of significant differences with respect to Pre-C values. The extinction of CPP must always be confirmed in a subsequent session performed 24 h after the last extinction session. We have observed that HNS mice show enhanced resistance to the extinction of drug-induced CPP (Fig. 6b).

To evaluate vulnerability to drug-induced reinstatement, the effects of a priming dose of the drug used to induce CPP—typically half of the dose administered during conditioning (0.0375 mg/kg of WIN 55212-2, 0.5 or 3 mg/kg of cocaine)—are evaluated 24 h after confirmation of extinction. Reinstatement tests are the same as for Post-C (free ambulation for 15 min), except that mice are tested 15 min after administration of the drug employed during the conditioning phase. Reinstatement tests are always performed in the dark phase, between 10:00 and 14:00 h. We have observed that HNS mice exhibit enhanced vulnerability to the extinction of drug-induced CPP (Fig. 6c).

2.3 Statistical Analysis

2.3.1 Hole Board

Mice are considered high novelty seekers (HNS) or low novelty seekers (LNS) according to whether their result is higher or lower than the mean of their group. The most usual measure is the frequency (number) of dips, although the latency to the first dip can also be used (*see* **Note 13**). Although the distribution is not completely bimodal (some mice had a similar number of head dips in LNS and HNS groups), they are clearly different with respect to the median score. To assess differences in the frequency of head-dipping between male adolescent HNS and LNS mice, a *t*-test is usually carried out. In all cases, this test reveals any significant differences between the two groups ($p < 0.01$).

In other studies, a mixed ANOVA is performed for each measure of the novelty-seeking test (number of dips and/or latency to the first dip). In function of the experimental design of the study, there is/are one, two, or three between-subject variables: *sex* (with two levels; males and females), *age* (with two levels; adolescents and young adults), or/and *pretreatment* (drug and saline). Sometimes, separate ANOVAs are performed for adolescent and adult animals with the variable "sex," with two levels (male and female), and for male and female animals with the variable "age," with two levels (adolescents and young adults).

Post hoc comparisons are performed with Bonferroni tests.

2.3.2 Conditioned Place Preference

For CPP, the time spent in the drug-paired compartment during pre- and postconditioning phases is analyzed by means of a mixed ANOVA with a *within variable—days*, with two levels (pre- and postconditioning test)—and one, two, three, or four between variables: *sex*, *age*, *pretreatment* (the same as explained in the previous section), and level of novelty-seeking (HNS and LNS).

Linear and logistic regression analyses may be employed to determine the association between the level of novelty-seeking and the development of CPP (using a CPP score, time spent in drug-paired compartment in Post-C minus time spent in the same compartment in Pre-C).

Differences between the time spent by mice in the drug- and saline-paired compartments in extinction and reinstatement tests after receiving priming doses are analyzed by means of Student's "*t*" tests. In some cases, the variable "days"—analyzed in the ANOVA described in the first paragraph of this section—has four levels: Pre-C, Post-C, extinction, and reinstatement. In addition, the time required for preference to be extinguished in each animal can be analyzed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate.

In the ANOVAs, post hoc comparisons are performed with Bonferroni tests.

3 Notes

1. The age of animals plays an essential role in the effects observed in the hole board test. In rodents, adolescence covers the whole postnatal period, from weaning (21 postnatal days, PND 21) to adulthood (PND 60). Rodent adolescence has been classified in three periods: early adolescence (prepubescent or juvenile, from PND 21–34), middle adolescence (periadolescent, from PND 34–46), and late adolescence (young adult, from PND 46–59) [150]. Behavioral traits observed in adolescent humans [34] have also been observed in animal models. In comparison with their adult counterparts, adolescent rodents show increased exploration and risk-taking behavior [31, 36, 39, 165]. Juvenile rodents present greater hyperactivity and exploration in novel environments [36, 166], perform a higher number of explorations in the novel object recognition task and novel environment test of free-choice [66], and display higher locomotor activity in the initial minutes of exposure to an inescapable novel open field [63, 113]. Adolescent mice are also prone to present shorter latency to approach novelty and to explore a novel object/environment for longer periods than their young adult counterparts. In some studies, the hole board has been applied to adult mice (>PND 60), namely, to observe

changes in their behavior induced by environmental manipulations (e.g., exposure to stress).

2. It is important to consider the age of animals when selecting the dose to be used in the CPP paradigm. Generally, conditioned reward is enhanced in adolescents in comparison to adults [39]. For example, we have observed a clear influence of age on the rewarding effects of some drugs of abuse. Adolescent male mice conditioned with ethanol on PND 32–38 showed CPP with doses (1.25 and 2.5 g/kg) that were ineffective in young adult animals conditioned on PND 54–60 [167]. In contrast, we have seen that 1 mg/kg of cocaine is effective in inducing CPP in both adolescent (PND 29–32) and late adolescent (young adult) mice (PND 50–53), although the latter group showed reinstatement of the CPP induced by 25 mg/kg of cocaine with lower priming doses of this drug (12.5, 6.25, and 3.125 mg/kg) than early adolescent mice, in which reinstatement was achieved only with 12.5 mg/kg of MDMA [168]. In the case of MDMA, late adolescent mice are more sensitive to it's rewarding effects than early adolescent mice (PND 53–59 vs. PND 32–38, respectively, during conditioning). A low dose of MDMA (1.25 mg/kg) induces CPP in late adolescent mice only; furthermore, the CPP induced by 10 mg/kg of MDMA is reinstated in both age groups by 5 mg/kg of MDMA, but only late adolescent mice show further reinstatement with 2.5 mg/kg of MDMA [169].

   The influence of the novelty-seeking trait on drug-induced CPP also depends on the age of the animals. Previous reports by our group have shown that naïve HNS adolescent mice acquire CPP after conditioning with 1 mg/kg of cocaine, a dose that is ineffective in LNS animals [66, 114]. Conversely, when male adult rats are classified as HNS versus LNS, neither group develops CPP with 2.5 mg/kg of cocaine [107].

3. Sexual differences in the effects of drugs of abuse on the CPP paradigm have been reported; for instance, female rats develop CPP at lower doses than males [145]. Similarly, we have observed that ethanol induces CPP and reinstatement in young adult female mice at doses (2.5 g/kg) that are ineffective to induce these effects in male mice of the same age [167].

4. In CPP experiments we usually handle mice on each of the 3 days immediately prior to the preconditioning (Pre-C) phase in order to reduce their stress levels in response to experimental manipulations. Prevention of noise, a quiet environment throughout the laboratory and the habituation of animals to the room where the behavioral tests are performed is also critical if reliable results are to be achieved.

5. For CPP experiments, we employ a subthreshold dose of cocaine (or other drugs of abuse) in order to determine the sensitizing effect of adolescent drug treatments on the conditioned reinforcing effects of cocaine. If a dose that is ineffective in naïve mice can induce CPP in mice preexposed to a drug of abuse during adolescence, it can be assumed that the adolescent treatment has enhanced the vulnerability of mice to the rewarding effects of this drug. In other cases, to evaluate if adolescent drug exposure increases the probability of relapse to drug use after a period of abstinence, we administer a dose that induces CPP in naïve mice but does not produce reinstatement after drug priming (e.g., 6 mg/kg of cocaine) [66, 157].
6. Cannabinoid drugs are difficult to dissolve in physiological saline. It is recommendable to first mix the cannabinoid with a quantity of DSMO (for example, 3 mL) and a drop of Tween 80. Physiological saline is then added bit by bit. An electric shaker may be used to facilitate dissolution.
7. Intraperitoneal (i.p.) injection is an easy and effective way to administer drugs to mice (*see* Fig. 2). It is important to use a new needle for each animal (to reduce discomfort and risk of infection), although in the case of chronic treatments the syringe can be marked and maintained for later use in the same animal.
8. The novelty-seeking test must be performed at least 1 h after initiation of the dark phase of the cycle, since mice are nocturnal animals whose circadian phase of activity takes place at night. For the same reason, the experimental room should be only slightly illuminated.
9. It is recommended to place mice in the same corner of the cage at the beginning of the novelty-seeking test and it should be verified that a mouse of medium size cannot pass through the hole. Moreover, there are different frames with holes for rats or mice. This is especially important in the case of adolescent mice of some strains that are very small.
10. In a previous study carried out in our laboratory, we reported a higher predictive capacity of the hole board test for identifying "drug-vulnerable" individuals among adolescent mice of both sexes. HNS mice acquired CPP after conditioning with 1 mg/kg of cocaine, while LNS mice did not [114].
11. Our protocol of cocaine administration was adapted from that of Sillivan et al. [170] and has proved to induce long-lasting alterations in rodent behavior [170, 171]. It should be stressed that we use lower doses of cocaine to administered binges to mice that are subsequently to be conditioned with cocaine or MDMA, since we have observed that pretreatment with high

doses of cocaine decreases the subsequent rewarding effects of this drug [172].

12. Animals showing strong unconditioned aversion for any compartment (less than 33% of the session time) must be excluded from the rest of the protocol to ensure that the CPP procedure is unbiased in terms of initial spontaneous preference [157, 159]. Therefore, we determine which compartment will be paired with the drug (and which will be paired with the vehicle) for each mouse. We use a counterbalanced design by which half the animals in each group receive the treatment in one compartment (black) and the other half receive it in the other (white) compartment.
13. Latency data undergo logarithmic transformation before statistical analysis.

4 Advantages and Limitations of the Hole Board Test When Employed to Predict Vulnerability to Drug Addiction

A major limitation of studies in human beings is the impossibility of establishing causal relationships between events or facts. In the case of the novelty-seeking trait, we cannot determine whether a high novelty-seeking profile leads an individual to progress from recreational to compulsive drug consumption, or whether this behavioral profile is the consequence of drug abuse. Some studies have suggested that high novelty seekers have a differential sensitivity to drug reward at initiation of consumption that increases their vulnerability to develop addictive behavior [12, 173–176]; however, other studies challenge this putative relationship between sensation/novelty-seeking and addiction risk [18, 177–179]. In particular, it has been suggested that high novelty seekers are more prone to initiate and maintain a regular drug use, but that, without additional risk factors (such as a family history of addiction), they do not run an enhanced risk of developing drug addiction [179].

Animal models avoid the ethical problems of research with humans and have demonstrated themselves to be useful tools for increasing our understanding of the interacting factors that facilitate the development of drug addiction, including individual traits (such as a high novelty-seeking profile) and environmental events (e.g., early drug exposure or stressful experiences). In this sense, the hole board test allows us to assess the causality between a novelty-seeking profile and vulnerability to the development of addiction-like behavior in rodents. Another advantage of the hole board test is the validity of the model, since there is a close similarity between the behavioral patterns, physiological correlates, and psychobiological consequences observed in rodents and human beings with the novelty/sensation-seeking trait [23, 180].

The paradigm that we have described here allows researchers to study the interaction between two main factors that increase vulnerability to repeated drug consumption, that is, the behavioral predisposition of a high novelty-seeking profile and exposure to drugs of abuse during adolescence. The HNS phenotype may increase the probability that an adolescent will initiate drug experimentation and develop neuroadaptations that will lead later on to addiction in adulthood. Our research in mice has shown that the NS trait facilitates acquisition of the CPP induced by different drugs of abuse. Furthermore, we have observed that the HNS trait increases the effects of exposure to drugs of abuse during adolescence on the reinstatement and maintenance of cocaine CPP. Thus, adolescent exposure to psychostimulants, EtOH or cannabinoids induces more profound effects on the behavior of subjects with an HNS profile and their response to drugs of abuse. From a translational point of view, our paradigm models a subpopulation of adolescents which engages in drug use early in life and has a greater risk of developing abuse and addiction. Thus, there is no direct causal relationship between adolescent drug exposure and the later development of addiction; rather, our results suggest that there are subjects with particular behavioral traits, present prior to the onset of drug use, that are more vulnerable to addiction. For example, subjects with a higher propensity for sensation-seeking, in addition to a greater tendency to experiment with drugs, are likely to develop more neuroadaptations following drug exposure, thus resulting in the transition from voluntary to compulsive drug use.

Some of the limitations of our model are related to what the hole board and the CPP paradigms are really measuring. Besides novelty-seeking, the hole board test can be used to assess emotionality and anxiety in mice [79, 81]. In addition, the enhanced ability of HNS mice to develop CPP with low doses of psychostimulants or cannabinoids may be due to different factors; in comparison with LNS mice, those with an HNS profile may experience the drug as more rewarding, may enhance the attribution of incentive salience to the drug-associated cues and/or may acquire conditioned learning more efficiently.

The influence of the novelty-seeking trait on the rewarding effects of drugs of abuse needs to be studied in adolescent animals because novelty-seeking behavior and drug sensitivity are more pronounced in adolescent than in adult rodents. However, the influence of the novelty-seeking trait on drug consumption has generally been studied in adult rodents [12]. One limitation of the hole board is the fact that adolescent mice show a lower index of novelty-seeking (measured by the number of dips) than young adult mice (*see* Table 1); nevertheless, we have selected this model of novelty-seeking because it is the only one capable of predicting enhanced vulnerability to cocaine-induced CPP in HNS adolescent

male mice. As of yet we have not evaluated sex differences in the way the novelty-seeking trait modulates the effects of adolescent drug exposure on the later development of addiction.

5 Future Directions and Conclusion

One priority of future research should be the identification of neurobiological substrates of the HNS profile and the associated vulnerability to drug addiction. Dopamine is the neurotransmitter most related with this behavioral trait. HNS animals present higher endogenous levels of dopamine, lower availability of D2/D3/D4 receptors and stronger responses to rewarding stimuli and reward-associated cues [181]. This characteristic striatal DA profile of HNS mice may contribute to their tendency to approach novel stimuli and to acquire drug-induced CPP with lower doses than LNS mice. Further knowledge of the role of other neurotransmitter systems and brain areas involved in novelty-seeking will no doubt contribute to the design of pharmacotherapies that reduce the risk of addiction in subjects with a more vulnerable behavioral profile.

A recent study in our laboratory has demonstrated that the novelty-seeking trait is a behavioral marker of vulnerability or resilience to the effects of stress on drug reward. In particular, young adult C57BL6 male mice suffering social stress (induced by exposure to repeated social defeat) are more sensitive to the effects of cocaine in the CPP paradigm during adulthood. However, we also observed that the long-term effects of social defeat were modulated by the behavioral response of mice to stress. Mice performing more dips in the hole board 24 h after the last defeat were later more vulnerable to the effects of stress on CPP; in fact, defeated HNS mice developed place conditioning with low doses of cocaine (which were ineffective in control and defeated LNS mice). The fact that defeated mice performing a lower number of dips in the hole board do not develop cocaine-induced CPP suggests they are resilient to the effects of stress. Future studies need to explore the interaction between the different variables that participate in vulnerability or resilience to stress.

Finally, as commented on before, sex differences in hole board behavior have been studied very little. Future studies should evaluate how novelty-seeking and other personality and environmental factors differentially modify the long-term effects of adolescent drug exposure in male and female subjects. The knowledge obtained in the following years will be of vital importance for drawing up the guidelines of specific preventive programs aimed at more vulnerable subjects.

In conclusion, our studies suggest that the high novelty-seeking endophenotype is a marker of susceptibility to the effects of environmental variables, such as adolescent drug exposure or

stressful experiences, and that it increases the risk of drug addiction. Advances in knowledge of such endophenotypes will constitute the scientific basis for the development of new preventive strategies and effective individualized therapies aimed at individuals at risk of addiction that reduce drug consumption and mitigate this disorder.

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Chapter 8

María Pilar García-Pardo, Jose Enrique De la Rubia-Ortí, **Claudia Calpe-López**, María Angeles Martínez-Caballero, María A. Aguilar. Influence of social defeat stress on the rewarding effects of drugs of abuse. In: Methods for Preclinical Research in Addiction, Neuromethods, vol. 174, María A. Aguilar (ed.), https://doi.org/10.1007/978-1-0716-1748-9_8, © Springer Science+Business Media, LLC, part of Springer Nature 2022.

Chapter 8

Influence of Social Defeat Stress on the Rewarding Effects of Drugs of Abuse

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Abstract

Drug addiction is a serious problem in our society. Some individuals develop dependence to different substances very accessible in the market, mainly young people. It is known that different biological and environmental variables facilitate the initiation, maintenance and relapse to drug use. In this sense, social stress is an important factor involved in the development of drug addiction and animal models are an optimal tool to study neurobiological systems associated with stress and addictive disorders. Among the main paradigms of social stress, the social defeat in an agonistic encounter with a conspecific male rodent has a notable ethological validity. Two main procedures, "acute" or "repeated" social defeat, may be distinguished, being the main differences between both the duration and intensity of the social defeat episodes and the evaluation of their short/long-term effects. Indeed, it has been demonstrated that the effects of both types of stress on the self-administration or conditioned place preference induced by different drugs are different. Although acute and repeated social defeat procedures have some limitations, in general terms both paradigms can help us to draw conclusions about the relationship between stress and drug addiction.

Key words Acute social defeat, Repeated social defeat, Reward, Drug, Stress, Animal models

1 Introduction

Drug addiction is a serious problem in our society. It is defined as a mental disorder according with diagnostic criteria on DSM-5 that involved emotional and behavioral aspects [1, 2]. Some people developed dependence to different drugs and even after long periods of abstinence relapse can appear. It is known that an important variety of addictive substances are available in the market

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[3]. Substances such as alcohol, cannabis, psychostimulant, or opioid drugs are heavily consumed mainly between young people [4].

Animal models are essential for the identification of factors that induce vulnerability to drug addiction, which is important in order to develop preventive and treatment strategies for this disorder. Some studies have evidenced that drug addiction is associated with biological variables, for example, genetic aspects [5]. Furthermore, several studies show that environmental factors have a profound influence on the acquisition, maintenance, and relapse to drug addiction [6–9]. Between these environmental factors, stress can be determinant not only in the transition from drug use to abuse but also in the development of mental and cognitive disorders related with drug addiction [7, 10]. A wide variety of stressful stimuli have been employed to model the influence of stress on the rewarding effects of drugs of abuse [11]. Some studies used pharmacological stressors (e.g., corticotropin-releasing factor CRF) [12], physical (e.g., intermittent shock, immobilization, or tail pinch) [9, 13, 14], emotional (food deprivation, forced swim, the chronic unpredictable stress) [15–17], or social stress (maternal separation, social isolation, crowding, social defeat) [9, 18–20]. Among these types of stressors used in animal models, social defeat has showed high ethological validity. Fortunately, food deprivation or intermittent shocks are unlikely to be experienced nowadays. However, social adverse events, including bullying, are common in our society.

The association between stress and drug addiction seems to have a neurobiological explanation, since there is a nexus between the brain and the stress system. It is known that under stress the hypothalamus–pituitary–adrenal (HPA) is activated as a central control and regulatory system involved in the stress response together with the sympathetic-adrenal system. Moreover, the mesocorticolimbic dopaminergic system involved in drug reward [21, 22] has a close connection with the brain stress system [11]. In fact, the activation of brain stress system is key to induce the emotional adverse state that characterizes abstinence and leads to relapse to drug-seeking behavior [23]. The common nexus between stress and reward systems is the "extended amygdala," composed by the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA) and interconnected with the main brain areas of the reward system including the nucleus accumbens, the ventral tegmental area (VTA), and the prefrontal cortex (PFC). Several neurotransmitters involved in stress and reward such as CRF, noradrenaline (NA), and dopamine (DA) interact in the extended amygdala [11]. The PFC has an important role in the stress response, via attenuation of the activity of amygdala that process the emotional stimulus [24]. However, different types of stress can alter these brain regions in a distinctive way. As social stress does not represent an immediate or direct threat for the survival of the organism, this stimulus needs to be processed by the prefrontal cortex (PFC), a rational part of our

brain. A recent study demonstrated that the activity of individual neurons of the PFC is altered by social defeat stress [25].

Several animal models have been used to evaluate the rewarding effects of drugs of abuse, although the self-administration (SA) and the conditioned place preference (CPP) are the most commonly employed [26]. These paradigms also have allowed to evaluate the influence of stress in the rewarding properties of drug abuse [20, 27–29]. At the same time, different paradigms have been used to induce social stress although acute or repeated social defeat are the most used. Acute social defeat (ASD) is usually used to evaluate the short-term effects of social stress while the repeated social defeat (RSD) paradigm allow to observe its long-term effects. Both will be described in the next section.

This chapter is aimed to provide an update of studies about the role of social stress on the rewarding effects of drugs of abuse using animal models. First, we describe as social defeat modifies the rewarding properties of the most consumed drugs of abuse (alcohol, psychostimulants, cannabis, and opioids) using the SA and CPP paradigms. Next, we describe the materials and methods used to perform the ASD and RSD procedures in our laboratory. Finally, we draw the final conclusions and propose future lines of research with these paradigms of social stress.

2 Social Defeat Modifies the Rewarding Properties of Drugs of Abuse

2.1 Influence of Social Stress on the Rewarding Effects of Ethanol

Ethanol is the drug of abuse most typically consumed in our society. The study of the environmental variables that can modify its rewarding properties is essential in order to determine factors that can enhance the vulnerability to the abusive consumption and for the development of new behavioral therapies.

However, the studies about the effects of social stress on the rewarding properties of ethanol are controversial and sometimes depend on the paradigm of reward employed as well as on the strain or age of animals used [30]. In the ***SA paradigm*** it has been showed that mild social defeat stress is sufficient to increase alcohol consumption in nonpreferring strains of rats [31]. Similarly, exposure to RSD during adolescence increases vulnerability to the rewarding effects of ethanol in mice [32] and moderately stressed mice showed SA during intermittent access to ethanol and escalated intake [33]. Administered during a period of deprivation of alcohol, social defeat caused a smaller increase in alcohol intake but only after a first deprivation and stress cycle [34]. However, other studies reported that exposure to different patterns of social defeat could induce a transient suppression rather than a facilitation of ethanol intake [35, 36]. The interval between social stress and the evaluation of alcohol intake influences the results observed. A decrease of alcohol self-administration was observed 24 h after the previous social stress episode; however, no changes in intake or alcohol

reinforcements were observed 4 h after exposure of rats to social defeat [35]. Acute exposure to social defeat decreased alcohol self-administration, reduced rates of responding during extinction, and did not reinstate alcohol seeking [37]. Exposure to a discrete odor cue previously paired with social defeat also decreased alcohol self-administration but induced a modest reinstatement of alcohol seeking [37].

With respect to the results obtained with the ***CPP paradigm*** most studies support the idea that social stress increased ethanol-induced CPP [38–41]. In the same line we have observed that acute and intermittent/long-term types of social defeat stress (ASD and RSD) reversed the conditioned place aversion induced by high doses of ethanol [42]. In this study, we also evaluated the acute and long-term effects of social defeat on alcohol consumption in the two-bottle choice paradigm. Mice exposed to social defeat showed an increase in alcohol intake in comparison to control nonstressed mice [42].

2.2 Influence of Social Stress on the Rewarding Effects of Psychostimulants

Cocaine and "ecstasy" or MDMA (3,4-methylenedioxymethamphetamine) are psychostimulant drugs widely consumed in our society [4]. Different studies have evaluated the influence of social defeat stress on their rewarding properties.

Using the SA paradigm Several studies have demonstrated that social defeat increased vulnerability to acquiring and maintaining **cocaine** self-administration and prompts an escalation of cocaine-seeking behavior [43–51], probably the VTA DA neurons and CRF being involved in this effect [52]. Moreover, it seems that behavioral characteristics during social defeat are predictive of later cocaine self-administration [53]. On the other hand, intermittent social defeat did not modify cocaine self-administration in mice [54] or modestly increased it [55]. Individual differences have been observed in the effects of continuous exposure to social defeat; it increased cocaine self-administration and sucrose intake in a subset of animals, but decreased these measures in another subpopulation of mice. Acquisition of cocaine self-administration (0.5 mg/kg per injection) was delayed in adult mice exposed to RSD during adolescence [56].

Social defeat potentiated methamphetamine seeking. In rats trained to acquire the self-administration of methamphetamine and that subsequently underwent extinction of lever pressing, a single social defeat did not reinstate drug seeking; however, a reminder of social defeat followed by a priming injection of methamphetamine potentiated reinstatement over drug-priming alone [57].

No study has evaluated the role of social stress on the rewarding properties of MDMA using the SA paradigm, so more studies are needed in this area.

Using the CPP paradigm It has been evidenced that social defeat stress modifies the rewarding properties of cocaine (see the review of Montagud-Romero et al. [30]). Mice exposed to social defeat exhibited an increased place-preference for the cocaine-paired chamber over the control group and the pretreatment with a kappa opioid receptor antagonist blocked this stress-induced potentiation of cocaine-CPP [58]. The effects of social defeat on drug reward are in function of several variables such as the age of the animals when are exposed to stress, the social stress schedule and the dose of drug used [59]. A single exposure to social defeat before the reinstatement test increased the vulnerability to reinstatement induced by a cocaine priming [19] and induces reinstatement of cocaine CPP [60, 61]. Adult mice exposed to ASD showed an increase in the conditioned rewarding effects of cocaine [28] and in the reinstatement of cocaine CPP [62]. Conversely, adolescent mice exposed to ASD display a reduction of the conditioned rewarding effects of cocaine. In fact, CPP was not observed with a low dose of cocaine (1 mg/kg) that is effective to induce CPP in adolescent mice non exposed to social defeat; furthermore, when adolescent mice were conditioned with high doses (25 mg/kg), the CPP extinguished faster in those exposed to social defeat [28].

With respect to the long-term effects of RSD, an enhancement in the rewarding effects of cocaine is observed in both adult and adolescent mice. Three weeks after exposure to RSD in adulthood, mice showed CPP with a low dose of cocaine (1 mg/kg) that is ineffective to induce place conditioning in control mice [63–68] This increase in the rewarding effects of cocaine induced by RSD is mediated by an upregulation of histone acetylation induced by social defeat [63], by DA neurotransmission [64] and is reversed by indomethacin [66], the antagonism of CRF1 receptors [67] and oxytocin [68]. Furthermore, the RSD-induced increase of cocaine CPP is observed regardless of the genotype of mice [69]. Similarly, animals socially defeated during adolescence showed an increase in the conditioned rewarding effects of cocaine in the adulthood [56, 70]. Such increase is expressed as an enhanced vulnerability to the reinstatement of the CPP induced by a low dose of cocaine (1 mg/kg) in mice defeated during adolescence and as a prolonged duration of the CPP induced by 25 mg/kg of cocaine, since defeated mice needed more sessions for the preference to be extinguished [56]. It is especially relevant since adolescent mice experienced social defeat less intensely than adults and showed lower levels of corticosterone.

With respect to **MDMA**, most studies have showed that social defeat modified the conditioned rewarding properties of this drug, although as commented before with cocaine, the results obtained depend on different variables [59]. For example, age is an important factor for the effects of ASD. Exposure to defeat in agonistic

encounters with an aggressive conspecific immediately before each session of place conditioning does not induce alterations on the rewarding or other behavioral effects of MDMA (1.25–10 mg/kg) in adolescent mice. However, in young adult mice exposure to the same type of defeat decreases the sensitivity of adult mice to the rewarding effects of MDMA since defeated mice did not show CPP with doses (1.25–10 mg/kg) that are effective to induce CPP in control mice [27]. Changes in the glutamatergic system seem to play a role in the effects of social defeat stress on the rewarding properties of MDMA. In particular, the pretreatment with memantine (NMDA antagonist) to young adult mice exposed to an episode of ASD before each conditioning session with MDMA (1.25 mg/kg) reverse the effects of social defeat since defeated mice treated with memantine showed CPP [76]. Conversely, the pretreatment with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (AMPA antagonist) did not modify the effects of ASD since defeated mice treated with CNQX did not show CPP. The results of this study also indicate that social defeat induces modifications in the brain areas involved in the reward. Social defeat decreased the expression of several subunits of glutamatergic receptors NMDA and AMPA, for example, GluN1 and GluA1 [71]. A recent study also indicates the role of the nitric oxide (NO) pathway in the effects of ASD on the rewarding properties of MDMA. In particular, pretreatment with a low dose of the NO synthase inhibitor 7-nitroindazole (7.25 mg/kg) reversed the effects of ASD since defeated mice show CPP. Moreover, social defeat exposure increased the nitrites in the prefrontal cortex and hippocampus [72].

On the other hand, the long-term effects of social defeat on the conditioned rewarding effects of MDMA are consistent in adolescent and adult mice. Exposure to RSD 3 weeks before place conditioning with MDMA enhanced vulnerability to priming-induced reinstatement in mice conditioned with a low dose of MDMA (1.25 mg/kg) and increased the duration of CPP induced by a high dose (10 mg/kg) [20].

2.3 Influence of Social Stress on the Rewarding Effect of Other Drugs of Abuse

Few studies have evaluated the effects of social defeat stress on the rewarding properties of opioid drugs. Rats with a history of intermittent social defeat persisted in self-administering a heroin–cocaine mixture and showed escalated cocaine-taking behavior during the 24-h binge session, although no effects on heroin taking were observed [48]. On the other hand, social defeat prevented the acquisition of morphine CPP, which suggests a decrease in sensitivity to the rewarding effects of opiates [73]. However, social defeat in an agonistic encounter induced reinstatement of CPP in morphine-conditioned animals [14]. These data support the idea that social stress can modulate opiate reward and promote relapse [74].

Studies about the influence of social stress on the rewarding properties of nicotine or cannabinoid drugs are inexistent.

3 Main Paradigms Used to Induce Social Defeat Stress

As we have described in the previous sections, social defeat is an animal model of stress exposure with high ethological and ecological validity. However, we used two main variations of the paradigm (acute and repeated social defeat) that differed in function of several factors, mainly the number of exposures to defeat experience, the duration and context of the agonistic encounters and the time elapsed between the stress experience and the exposure to drugs of abuse (*see* Table 1).

3.1 Acute Social Defeat

3.1.1 Materials

- *Experimental animals*: We use male OF1 mice (Charles River, France) of 21 days or 42 days of age at the arrival to the laboratory (adolescents and young adults, respectively). They are housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 8 days before the experiments began (*see* **Note 1**).
- *Aggressive Opponents*: We use male OF1 mice of 42 days of age at the arrival to the laboratory. They were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before experiments to induce aggression [75] (*see* **Note 2**).

Table 1
Differences between acute social defeat (ASD) and repeated social defeat (RSD)

	Objective	Duration and environment	Number of episodes	Evaluation of rewarding properties of drugs	Animals used
Acute social defeat (ASD)	Evaluate the **short-term** effect of social defeat	The agonistic encounters are performed in a **neutral box** for **10 min.**	**Only** one episode of social defeat.	**Immediately**, before each conditioning or self-administration or reinstatement.	Mice and rats Adults and adolescents
Repeated social defeat (RSD)	Evaluate the **long-term** effect of social defeat	The agonistic encounters are performed in the **resident home cage** for **25 min.** Only 5 min of interaction.	**Four** episodes of social defeat each 72 h separated in three phases each of them.	**Three weeks** after the last social defeat.	Mice and rats Adults and adolescents

- *Cage for the agonistic encounters*: We use a neutral transparent plastic cage (23 × 13.5 × 13 cm), that is to say, an area that is not the home cage of the experimental or the aggressive animal.
- *Video camera*: The agonistic encounters are videotaped for the behavioral analysis.
- *Computerized program* (Raton time) and computer with screen and keyboard for behavioral analysis.

3.1.2 Methods

1. *Conditions of housing*: All mice are housed in a room under constant temperature, a reversed light schedule (white lights on 19:30–07:30 h) (*see* **Note 3**) and food and water freely available, except during behavioral tests.
2. *Induction of aggressiveness in the opponents*: As commented before, to ensure that opponents showed aggressive behaviors, they live isolated (*see* **Note 2**) and are briefly confronted with other isolated mice to instigate threat and attack behaviors.
3. *Screening of aggressive behaviors in opponents*: The opponent animals, which had previous fighting experience, are screened for a high level of aggression (with short latency to show threat and attack behaviors) in a brief encounter with a grouped conspecific (*see* **Note 4**). This screening encounter ends after the presence of threat and the first attack of the opponent and is performed typically 24 h before the agonistic encounter of social defeat with the experimental mouse.
4. *Agonistic encounter with the result of defeat for the experimental mouse*: The agonistic encounter between the experimental mouse and the aggressive opponent (*see* **Note 5**) takes place in a neutral transparent plastic cage (*see* Fig. 1a). First, animals are placed in this cage separated by a transparent plastic barrier during 1 min. Then, this barrier is removed and the physical interaction between them is allowed for 10 min. In response to the aggressive behaviors of the opponent, experimental animals (that are not housed in isolation and have not fighting experience) exhibited avoidance/flee and defensive/submissive behaviors. The criteria used to define an animal as defeated is a specific posture, characterized by an upright position, limp forepaws, upwardly angled head, and retracted ears [76] (*see* **Note 6**). The agonistic encounters are video recorded to subsequently evaluate the behaviors of both animals (*see* **Note 7**).
5. *Behavioral analysis of agonistic encounters*: The behavioral acts and postures showed by mice during the confrontations are recorded, in particular, the frequencies, durations, latencies, and temporal and sequential patterns of the different behavior (ethogram). Submissive and fleeing behaviors of the experimental animals and the aggressive behaviors of the opponents

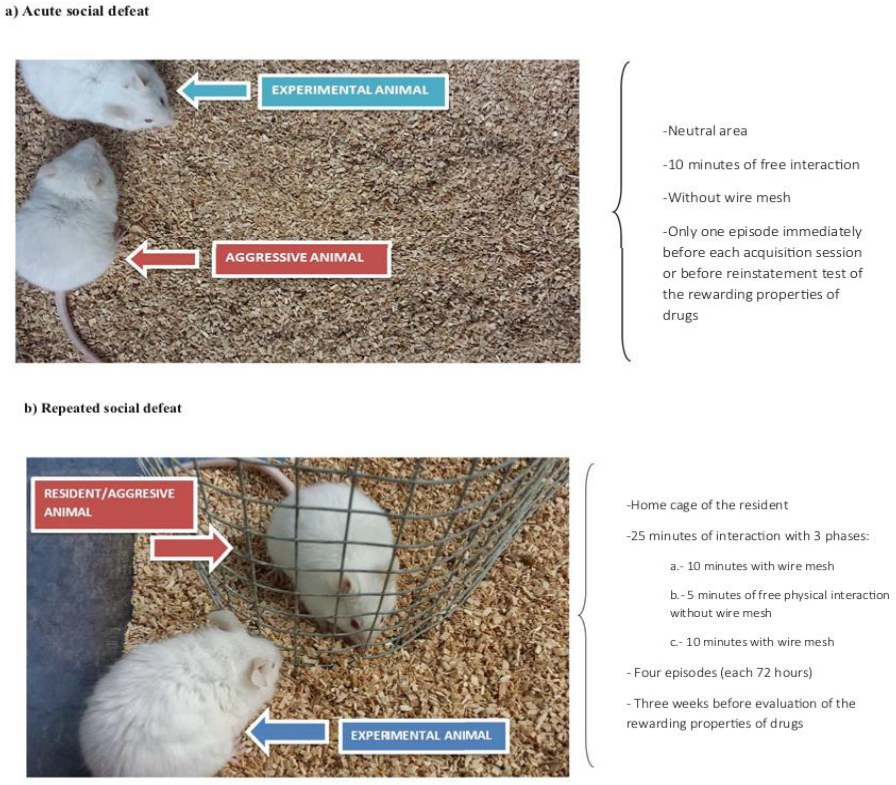

Fig. 1 Methodology of Acute social defeat (ASD) versus Repeated social defeat (RSD). (**a**) Acute social defeat. (**b**) Repeated social defeat

are evaluated using a custom-developed program that allows estimation of the time engaged in different behaviors (mainly threat, attack, avoidance/flee, and defense/submission) [75] (*see* Fig. 2 and Table 2) (*see* **Note 8**).

6. *Control group without defeat*: We used a control group of mice that do not suffer stress. In this case, the experimental animal is placed in the neutral area with the barrier for 1 min and when the barrier is removed it explore freely the cage for 10 min without any aggressive opponent mouse [27].
7. *Evaluation of corticosterone levels*: Immediately after the first and fourth agonistic encounter, we obtained blood samples from the mice exposed to social defeat (stress group) or

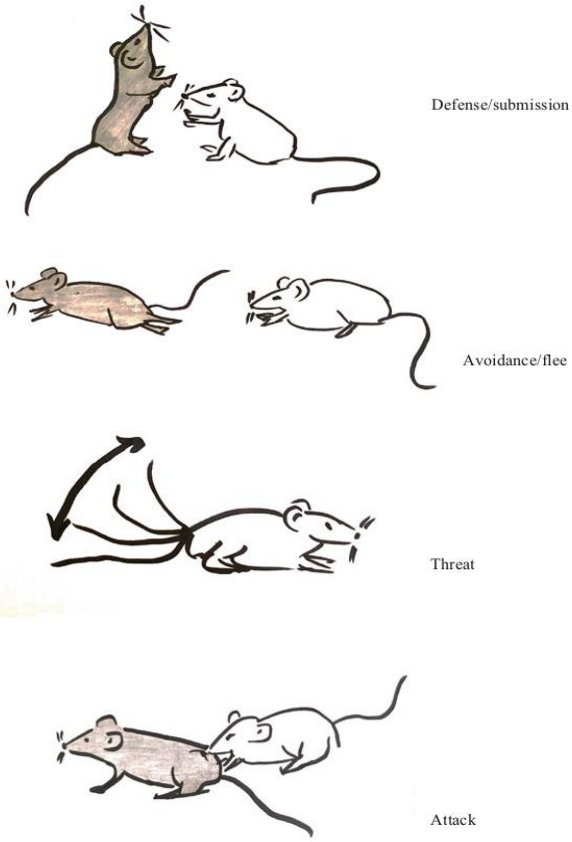

Fig. 2 Main behavioral categories recorded during episodes of defeat in the intruder mouse (in brown) and the aggressive opponent mouse

exploration of a new cage without opponent (control group) for corticosterone determination (*see* **Note 9**). Blood sampling is performed using the tail-nick procedure. In this procedure, the animal is wrapped in a cloth and a 2-mm incision is made at the end of the tail artery. Then, the tail is massaged to facilitate blood collection that is performed in an ice-cold Microvette CB 300 capillary tube (Sarstedt, Nümbrecht, Germany). We collect 50 μL of blood. Tubes with blood samples are kept on ice until they are placed in a centrifuge apparatus. Centrifugation (5 min, 5000 × *g*) separates plasma from whole blood. Plasma is transferred to sterile, 2 mL microcentrifuge tubes,

Table 2
Main behavioral category, elements and description

Behavioral category	Elements	Description
Body care	Abbreviated groom	A single rapid wipe of the head or snout using the forepaws
	Self-groom	Licking of the fur on the flanks or abdomen or preening of the tail
	Wash	Forepaws licked and then stroked repeatedly over the head
	Shake	A brief, mild quiver of the body
	Scratch	Hindlimb used to scratch at the flanks
Digging	Dig	Forepaws used to direct sawdust to the rear of the animal
	Kick dig	Hindpaws used to kick sawdust backward
	Push dig	Forepaws used to push sawdust forward
Non-social exploration	Explore	Walking or running around the cage, not directed toward partner
	Rear	Bipedal posture, front part of the body and forepaws raised
	Supported rear	Rear with forepaws resting on the walls of the cage
	Scan	Side-to-side movement of the head, attention not directed to partner
Explore from a distance	Approach	Ambulation and attention toward the partner
	Attend	Attention directed toward the partner from a distance
	Circle	Cycles of approaching and leaving with no intervening activity
	Head orient	Head turned toward the partner
	Stretched attention	As in attend, except that the body and head neck craned forward
Social investigation	Crawl over	Both forepaws placed on the partner
	Crawl under	Head and anterior part of the body pushed underneath the partner
	Follow	Moving in close proximity to the partner as it walks around the cage
	Groom	Grooming the body of the partner using the mouth
	Walk around	Walking around the partner, in close proximity
	Investigate	Sniffing the body or tail of the partner
	Sniff	Sniffing the anogenital region of the partner
	Nose sniff	Sniffing the head or snout of the partner
	Push past	The animals moving in opposite directions come into lateral contact
Threat	Aggressive groom	Vigorous grooming of the immobile partner from a lateral position, using the teeth and the forepaws
	Sideways offensive	Tripedal posture oriented toward the partner forepaws nearest the partner being raised, eyes slitted and ears flattened
	Upright offensive	Bipedal posture oriented toward the partner, characterized by slitting of the eyes and flattening of the ears
	Tail rattle	Rapid lateral quivering or thrashing of the tail
Attack	Charge	Running rapidly toward the partner
	Lunge	body, as if to bite the partner, but failing to make contact with teeth
	Attack	Biting the partner
	Chase	Pursuing a fleeing partner

(continued)

Table 2
(continued)

Behavioral category	Elements	Description
Avoidance/flee	Evade	Move anterior part of the body and/or head away from the partner
	flinch	Head rapidly retracted from the partner
	Retreat	Running away from approaching partner
	Ricochet	Fast undirected movement, with jumping from the walls of the cage
	Wheel	Turns as opponent approaches
	Startle	Sudden vertical movement in response to approach of partner
	Jump	Animal jumps into the air, all four feet leaving the substrate
	Leave	Ambulation away from the partner
	Wall clutch	Ventral surface of the body pressed against the cage wall, with forelimbs widely splayed
Defense/ submission	Upright defensive	Bipedal postures, usually oriented toward the partner accompanied by widening of the eyes and raising of the ears
	Upright submissive	An extreme form of "upright defensive" with the head pushed far backward and with the forelimbs held rigid and widely splayed
	Sideways defensive	Tripedal postures, one forepaw raised from the ground, accompanied by widening of the eyes and raising of the ears
Sexual	Attempted mount	Attempts to dorsally mount the partner, a motion which is incomplete
	Mount	Climbing dorsally on partner, palpating the flanks, and pelvis thrusts
Immobility	Squat	Complete immobility, no movements of any part of the body
	Cringe	As squat, but the body is pushed against the cage wall, animal pulls itself away from the partner and may make quivering motions

which are stored at −80 °C until determination of corticosterone. To perform the assay of corticosterone levels we use a corticosterone EIA kit (Catalog No. ADI-900-097, 96 Well kit; Enzo Life Sciences, Taper SA, Madrid, Spain). According to the manufacturer's instructions, plasma samples are diluted, in a proportion of ~1:40, in the Steroid Displacement Reagent mix provided with the kit. Corticosterone levels in diluted plasma are analysed using an iMark microplate reader and Microplate Manager 6.2. software (Bio-Rad, Madrid, Spain). The optical density was read at 405 nm, with 590 nm correction.

8. *Effects of acute social defeat on the rewarding effects of drugs of abuse*: In this paradigm of acute social defeat, as its name indicated, the experimental animal suffers a short and punctual experience of social defeat (10 min). When we used this paradigm in order to evaluate the effects of social defeat on the acquisition of the conditioned place preference induced by drugs of abuse, such as alcohol, cocaine or MDMA [20, 27,

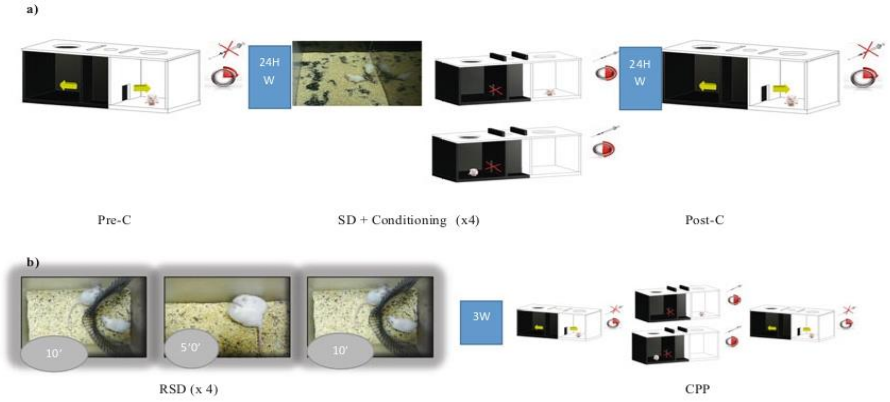

Fig. 3 Schedule of defeat episodes and CPP procedure. (**a**) Acute social defeat. (**b**) Repeated social defeat

28, 59, 71, 72] experimental mice suffer an experience of social defeat immediately before each conditioning session with the drug (thus, the agonistic encounter is performed only on 4 consecutive days or in alternative days, depending the drug used). Few hours after (24 or 48 h in function of the drug used) the effects of social defeat on the acquisition of place conditioning is evaluated (*see* Fig. 3a). On the other hand, to test the effects of social defeat on the reinstatement of CPP, experimental mouse only performed one agonistic encounter of 10 or 15 min with result of defeat, immediately or 30 min, before the reinstatement test [14, 19, 61].

3.2 Repeated Social Defeat

3.2.1 Materials

Experimental animals: We use male mice of the OF1 or C57BL/6 strain (Charles River, France). See details about the age and housing of mice in Subheading 3.1.

Aggressive Opponents: We use male OF1 mice of 42 days of age at the arrival to the laboratory. They were individually housed in plastic cages (23 × 32 × 20 cm) for a month before experiments to induce aggression [74]. Please note that in this case the home-cage of the aggressive opponents is longer because we used a resident–intruder model to induce social defeat.

Wire mesh barriers: to separate the experimental and opponent mice during the first and last 10 min of social defeat encounters.

Video camera, computer and computerized program (see details in the Subheading 3.1).

3.2.2 Methods

Conditions of housing, induction of aggressiveness and screening of aggressive behavior in the opponents are the same described in the Subheading 3.1.

1. *Repeated intermittent social defeat*: In this case, the experimental mouse is defeated in the context of a "intruder–resident" paradigm of aggression, based on the fact that an adult male rodent will establish a territory when given sufficient living space. The experimental animal (intruder) is placed in the home cage of the opponent (resident) mouse. As a consequence of isolation and territoriality, the resident will show offensive aggression versus the unfamiliar male mouse intruding in its home cage. In response to the offensive attacks by the resident, the intruder will show defensive/submissive behavior.

   In order to minimize physical harm but maintaining the stressful effects, the intruder is protected from the resident's attack by a wire mesh barrier during the main part of the encounter (*see* Fig. 1b) and physically exposed to the resident only for a brief time. In particular, each episode of social defeat (25 min) consisted of three phases.

   (a) *First phase*: Initially, the experimental animal is introduced in the home cage of resident aggressive opponent for 10 min but both animals are separated by the wire mesh protecting the intruder from the attack (bites) of the resident animal. However, social interaction between both animals and species-typical threats from the aggressive resident, as provocation and instigation, are allowed [20, 59, 77].

   (b) *Second phase*: The wire mesh is removed and the direct confrontation between animals is allowed for 5 min. We consider that the experimental (intruder) was defeated because of the adoption of the upright submissive position (as described in the Subheading 3.1) for 5 s [75, 78]. This posture appears normally after 3–5 attacks by the resident.

   (c) *Third phase*: In this last phase, both animals are separated again with the wire mesh for 10 min and the intruder animal is exposed to provocation and threat behaviors from the resident animal.

   Mice are exposed to several episodes of social defeat, usually one episode per day for 10 consecutive days (chronic social defeat) or, as we performed in our laboratory, one episode each 72 h until a total of 4 episodes (intermittent RSD). An important question is that in each aggressive episode the experimental animal is exposed to a different aggressive animal. However, when we confront experimental C57BL/6 mice with OF1 residents, we always use the same opponent in order to reduce the aggressive contacts received by the small-size experimental

animal. The first and fourth defeat episodes are video recorded to subsequently evaluate the offensive behaviors (threat and attack) of the resident and the defensive/submissive and avoidance/flee behaviors of the experimental animal.

2. *Behavioral analysis of agonistic encounters*: *see* Subheading 3.1.
3. *Control group without defeat*: We used a control group of mice that do not suffer stress. In this case, the experimental animal is placed in a cage (equal to the home-cage of the resident) without any mouse during 25 min, with a wire mesh wall on the first and last 10 min [20, 79].
4. *Evaluation of corticosterone levels*: *see* Subheading 3.1.
5. *Effects of repeated social defeat on the rewarding effects of drugs of abuse*: In our laboratory, the main objective of the RSD paradigm is to evaluate the long-term effects of the exposure to an intermittent situation of social stress on the acquisition, extinction and reinstatement of the CPP induced by different drugs of abuse [20, 59, 71]. Furthermore, in some studies we have evaluated the long-term effects of RSD on other behaviors (anxiety) or learning and memory processes.

In particular, the experimental animals are exposed to four episodes of social defeat that lasted 25 min each, separated by 72 h [20, 32, 80, 81]. To model exposure to social stress during adolescence animals are exposed to RSD on PND 29, 32, 35, and 38, while to model stress in young adult mice they experience social defeat on PND 47, 50, 53, and 56. In both cases, 3 weeks after suffering the last episode of defeat stress, animals undergo the place conditioning procedure with cocaine, MDMA, or alcohol [20, 42, 79] (*see* Fig. 3b).

4 Notes

1. To reduce the stress levels of mice in response to experimental manipulations, they are handled for 5 min/day on each of the 3 days before initiation of the behavioral experiments.
2. Mice of the OF1 strain are more aggressive than mice of other strains (e.g., Balb/c or C57). In addition, a month of isolation heightens aggression in OF1 mice [75].
3. The light schedule was reversed in order to facilitate the performance of the experimental procedures in the dark phase of the cycle, because mice are more active on night.
4. Mice confronted with aggressive opponents in the screening of aggressive behaviors are not other finality (usually they are animals employed in previous studies of our laboratory).

5. Usually, experimental adult animals are exposed to a conspecific of equal age and body weight. However, when adolescent mice are employed as experimental animals, the size of the opponent is higher.
6. Defeated mice always exhibit an extreme form of "upright submissive" behavior [75] (*see* Fig. 2). Furthermore, usually we performed subsequently a more profound analysis of the behavior of experimental and opponent mice (see the list of behaviors and postures in Table 2). A detailed description of all elements can be found in Brain et al. [82].
7. Agonistic encounters (social defeat) take place in a different room of that used for place conditioning or other behavioral experiments.
8. Usually only the behavioral analysis of the first and fourth agonistic encounter is performed.
9. Usually, a separate set of mice divided into two groups (social defeat and control) is used only to evaluate corticosterone levels. In other cases, the same mice that are exposed to CPP after social defeat or exploration are used (in this case, blood samples are obtained immediately after the place conditioning session).

5 Advantages, Main Applications, and Limitations of Social Defeat

Social stress is highly prevalent in our society. An elevated number of people suffer this type of stress in multiple situations affecting their mental and physical health. As we commented in the introduction of this chapter situations such as bullying and mobbing are common in different social interactions. The study of the consequences, prevention, treatment or variables associated with these social adverse situations are necessary in order to understand the neurobiological processes that are involved in the social defeat situations.

However, the induction of social stress in humans is not ethical and the use of animal models help us to progress on this research area. Acute/repeated social defeat paradigms allow us to draw conclusions about the effects of social stress. Moreover, the simulation in the laboratory of social stress using defeat paradigms have high ecological and ethological validity. In human social stress situations, the individuals also suffer a subordination by others that are hierarchically in a superior social scale (e.g., the boss at work or the leader in a group in high school). So, the use of defeat animal models is a great advantage to study aspects of social defeat that in other cases would be impossible. For example, the study of neuroanatomical structures and neurobiological pathways involved

in the stress response is determinant in order to develop effective treatments for the consequences of stress-related disorders. Moreover, defeat paradigms allow us to understand the main variables that influence the acute/long-term effects of social defeat (duration, intensity, frequency, intermittency) in order to develop preventive strategies to decrease the negative consequences of social stress. Although we used mice, the ASD and RSD paradigms can be performed in different strain of rats [83, 84].

As we have commented around this chapter, drug addiction is a chronic affective-cognitive disorder for which currently there is no cure. Thus, the study of variables involved in the initiation and maintenance of this disorder and in the vulnerability to relapse after withdrawal periods is essential. In agreement with clinical observations, animal models of social defeat have demonstrated an intimate relationship between social stress experiences and enhanced vulnerability to drug addiction. Several models of social defeat modify the rewarding properties of different drugs of abuse and a clear long-term increase in the sensitivity to psychostimulant drugs has been observed in defeated animals [29, 30, 59, 85]. As commented before defeat paradigms allow us draw conclusions about the consequences of different types of stress, different drugs of abuse, strain, sex, and age of animals. Moreover, these paradigms have allowed advances in the knowledge of the brain changes involved in the effects of social stress on drug addiction vulnerability. In fact, studies in our laboratory have demonstrated that NMDA and AMPA glutamatergic receptors are involved in the effects of social defeat stress [71, 86] and that the NMDA antagonist memantine reversed the effects of social defeat on MDMA [71] and cocaine [86] reward. Social defeat also increased the level of nitrites in several brain areas and the inhibition of NO synthesis reversed the effects of defeat on MDMA CPP [72]. These results suggested that the manipulation of glutamatergic system and/or NO pathway could be a useful therapeutic tool for the treatment of stressed individuals dependent on psychostimulant drugs. Other neurobiological systems have been involved in the effects of social defeat. Dopaminergic receptors [64, 65], the oxytocin system [68], BDNF [65, 87], and inflammatory signaling [66, 69, 88] are involved in the long-term effects of RSD on cocaine reward. These studies may contribute to developing pharmacological treatments for stress and addiction-related disorders.

A disadvantage of social defeat stress is that this paradigm is not free from ethical concerns and the difficulty to distinguish between the effects of physical and emotional stress. This is more important when experimental animals are confronted to more aggressive conspecifics that repeatedly bites them, for example, adolescent mice defeated by adult opponents. In fact, physical interaction between the animals during the agonistic encounter is reduced to a minimum, until the experimental mouse displays the behavioral posture

of defeat. However, the main limitation of social defeat paradigms is the difficulty to translate the results observed in animals to the clinical settings. Animal studies involve relatively brief exposure to the aggressive opponent that are often not representative of natural conditions or the duration of social interactions. In laboratory animal studies all the variables and conditions are under control, thus, the situation is more artificial. However, in humans other noncontrolled variables can affect the stress response. For example, personality traits can modify the physiological, cognitive, and behavioral responses to stress. Some of these aspects are being studied in new research projects (see the next section).

6 Conclusions and Future Perspective

As described in this chapter social stress can increase the initiation and maintenance of drug abuse and the vulnerability to relapse. In this sense, animal models help us to draw these conclusions. Social stress is an environmental factor that modifies our brain and in consequence our behavior. Moreover, the neurobiological systems involved in social stress are intimately associated with the reward brain pathway and with the structures related with the development of drug addiction [11]. Studies with different animal models of reward evidence that social defeat modifies the rewarding properties of different drugs of abuse including ethanol, opioid or psychostimulant drugs. However, the different paradigms of social defeat (single, intermittent, or continuous) and the time in which their effects on drug reward are evaluated (short- or long-term after social defeat) may influence the results observed [20, 27, 28, 65]. Moreover, other factors, such as the type of drug used or even the dose, can also modify the effects of social defeat stress [59]. Finally, individual variables such as sex [89], age [59], and/or behavioral traits of the experimental animals [79] have a profound influence in the effects of social defeat. For example, mice with a more active coping strategy during the episodes of defeat (with lower defensive behaviors) and mice with lower novelty-seeking profile are resilient to the potentiating effects of social defeat on cocaine reward (they do not develop CPP with low doses of cocaine while defeat mice usually do it).

Animal models of acute/repeated social defeat stress have demonstrated to be an essential tool to the progress gained regarding knowledge of the underlying mechanisms of stress and addiction. The use of these models has the advantage of greater control of experimental variables, has provided much valuable information and possesses high predictive value. On the other hand, the main drawback to animal studies is that any model reproduces totally all aspects that composed human stress and addictive disorders. Future lines of research should be proposed to enlarge the knowledge

about the neurobiological substrates underlying the interaction between these disorders. As commented before, behavioral traits associated with resilience to the effects of RSD on cocaine reward have been identified [79]. Future studies using other types of social defeat (such as ASD), other drugs of abuse (e.g., MDMA or ethanol) and animals of different sex or age (e.g. female or adolescent mice) can be important in order to draw conclusion about the role of these factors in the effects of social stress.

On the other hand, although it seems that stress is a negative event, some studies have indicated that a short and punctual stress in early life can cause the individuals to be more resistant to overcome future adverse stress situations, a phenomenon named as "inoculation of stress" [90, 91]. In this sense, more studies are necessary to demonstrate the effects of inoculation stress (using a single social defeat or other stressful events) on the subsequent effects of social defeat on the rewarding properties of drugs of abuse in other periods of life.

Other novel line of research of our laboratory is the study of the influence of social stress in individuals with neurodevelopment disorders, in particular, with attention deficit hyperactivity disorder. In children and adolescents with previous problems the effect of social defeat on the posterior rewarding properties of the drugs of abuse may be different. Moreover, sometimes these patients are treated with drugs to reduce the symptoms of these neurodevelopment disorders. For example, methylphenidate (an amphetamine derivate) is used for the attention deficit hyperactivity disorder and will be interesting to study the effect of this treatment in early age on the posterior response to the drugs of abuse in subjects that suffer social stress.

Finally, future works should study the neurobehavioral substrates of resilience after social stress in order to develop interventions that increase resilience to develop drug abuse and addiction in individuals at a high risk of suffering from stress.

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