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# Inflammation as a pathway between social stress and enhanced sensitivity to cocaine

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*A mis padres, Vicente y Carmen, por todo*

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# RESUMEN AMPLIO EN LENGUA OFICIAL

La respuesta al estrés es una estrategia adaptativa que nos ayuda a movilizar recursos para enfrentarnos a situaciones que ponen en riesgo nuestra supervivencia (Ulrich-Lai y Herman, 2009). Sin embargo, en nuestra sociedad el estrés constante se ha convertido en un problema relevante. Vivimos en un entorno social complejo que nos obliga a enfrentamos persistentemente a estresores "cotidianos" (Pearlin y Skaff, 1995) como por ejemplo los problemas de conciliación entre la vida laboral y familiar, discusiones de pareja y problemas económicos (Almeida, 2005), que puede llegar a afectar a nuestro bienestar incluso más que eventos más intensos pero menos frecuentes (Serido y cols., 2004).

Esta exposición crónica al estrés puede tener un impacto negativo y duradero en nuestra salud, tanto física como psicológica (Hartsell y Neupert, 2017; Lupien, y cols., 2009; McEwen, 2004). De hecho, numerosos estudios en la literatura científica han relacionado el estrés crónico con algunas de las enfermedades más comunes de nuestra sociedad como son el cáncer (Krizanova y cols., 2016), la obesidad y la depresión (Ouakinin y cols., 2018). En cuanto a las adicciones, nuestro foco de interés, el estrés también jugaría un papel central, participando en todas las etapas del proceso adictivo como son el inicio, mantenimiento, escalada y recaída del consumo de drogas (Koob, 2008; Koob y Schulkin, 2018).

Desde una perspectiva neurocientífica la adicción se considera una enfermedad cerebral crónica, caracterizada por la pérdida de control en el consumo de una sustancia adictiva. Su origen se considera multifactorial, y en él participarían factores tanto ambientales como biológicos (Kalivas y Volkow, 2005). En este contexto, las experiencias estresantes se consideran factores de riesgo ambientales que interaccionarían con los factores biológicos promoviendo un incremento de la vulnerabilidad a desarrollar un trastorno por uso de sustancias (TUS) (Ruisoto y Contador, 2019). Además, algunas fases del proceso adictivo como la abstinencia, pueden en sí mismas promover una respuestas fisiológica y psicológica de estrés (Koob y Schulkin, 2018).

La adicción es una enfermedad grave que pone en jaque el funcionamiento humano en todos sus dominios (Birkeland y cols., 2018), por tanto, resulta fundamental desarrollar estrategias que permitan controlar el impacto del estrés crónico sobre la vulnerabilidad a

desarrollar un TUS. Para poder desempeñar esta labor, primero es necesario determinar cuál es la principal fuente de estrés a la nos enfrentamos a diario. A pesar de que los estresores físicos, como por ejemplo el calor, pueden ser realmente molestos, la literatura muestra que los estresores provenientes de la interacción social son, para los humanos, la fuente más significativa de estrés (Dickerson y Kemeny, 2004; Tamashiro y cols., 2005). Un ambiente social positivo, con relaciones interpersonales estables, puede actuar como un reforzador alternativo al consumo de drogas, siendo un factor de protección para el desarrollo de TUS. Sin embargo, experiencias negativas como el aislamiento o el *mobbing* en el trabajo se han relacionado con mayores prevalencias de abuso de sustancias y una mayor vulnerabilidad a la recaída en el consumo de drogas tras periodos de desintoxicación (Heilig y cols., 2016; Niedhammer y cols., 2010; Sullivan y cols., 2006). Sin embargo, a pesar de que sabemos que el estrés social es central en la aparición y progresión de la adicción, en la actualidad desconocemos exactamente qué bases neurobiológicas subyacen a estos efectos del estrés social sobre el consumo de sustancias (Ruisoto y Contador, 2019).

En este sentido, la investigación básica en adicciones con modelos animales se ha mostrado útil para estudiar los efectos del estrés en la respuesta a las drogas. En general, la literatura científica muestra que experiencias de estrés social producen un incremento en la respuesta al refuerzo de las drogas, pudiendo además promover la reinstauración de la búsqueda de la droga en modelos de vulnerabilidad como son el condicionamiento de la preferencia de lugar (CPL) (Montagud-Romero y cols., 2018) o la auto-administración (Rodríguez-Arias y cols., 2016, 2017).

De estas investigaciones han emergido diversas teorías explicativas, que han relacionado las consecuencias del estrés con alteraciones en diversos sistemas cerebrales como por ejemplo alteraciones en el sistema de neurotransmisión del factor de liberación de la corticotropina (CRF) (Zorrilla y cols., 2014), alteraciones en el sistema de la dopamina (Reguilón y cols., 2017) o cambios neuroplásticos y epigenéticos (Montagud-Romero y cols., 2016). Recientemente estudios básicos han encontrado que el estrés social es capaz de activar el sistema inmune promoviendo un estado inflamatorio, tanto a nivel periférico como dentro del sistema nervioso central (SNC) (Hodes y cols., 2014; Pfau y Russo, 2016). Esta activación del sistema inmunitario por estrés se ha considerado un posible mecanismo mediante el cual se incrementaría la

vulnerabilidad a padecer ciertas enfermedades mentales, especialmente trastornos del estado de ánimo (Cathomas y cols., 2019).

De hecho, a nivel preclínico, la relación entre los estados inflamatorios inducidos por estrés y la enfermedad mental está bastante bien caracterizada en modelos animales de ansiedad y la depresión (Hodes y cols., 2016; Ménard y cols., 2017ab). En relación a los TUS, el consumo de ciertas sustancias ha mostrado producir alteraciones en el sistema inmune (Clark, y cols., 2013; Cui y cols., 2014), y procesos de inflamación del SNC que se han relacionado con la toxicidad y el deterioro cognitivo de algunas drogas, especialmente el etanol (Coller y Hutchinson, 2012; Hutchinson y Watkins, 2014; Montesinos y cols., 2015; 2016).

Mientras que los efectos inflamatorios del estrés y su impacto en los trastornos depresivos han sido ampliamente estudiados, en la actualidad ningún estudio ha analizado si procesos inflamatorios median también en el incremento en la vulnerabilidad a desarrollar TUS. Por tanto, el objetivo principal de la presente Tesis Doctoral será validar la hipótesis inflamatoria de la vulnerabilidad inducida por estrés en la adicción a la cocaína.

Para acercarnos a este objetivo, primero deberemos confirmar si el estrés social posee un potencial inflamatorio. Una vez demostrado quedará determinar su rol en el incremento de efectos reforzantes de la cocaína. Para ello exploraremos si intervenciones, tanto farmacológicas como conductuales, son efectivas bloqueando el efecto del estrés sobre el incremento a largo plazo en la respuesta a la cocaína.

En cuanto a la metodología empleada destacamos el paradigma de la derrota social (DS) como modelo de inoculación de estrés social en roedores. Este paradigma es ampliamente utilizado para modelar estresores sociales en roedores, siendo considerado el modelo más representativo para modelar las relaciones típicas de subordinación y agresión en humanos (Björkqvist, 2001; Hammels y cols., 2015; Neisewander y cols., 2012; Selten y cols., 2013).

La evaluación del perfil inflamatorio de los roedores se realizó mediante la estimación en plasma o tejido cerebral de la concentración de la citoquina pro-inflamatoria IL-6 mediante inmunoensayo. Para evaluar el efecto de estas experiencias estresantes en la

respuesta individual a la cocaína utilizamos el paradigma del CPL el cual permite evaluar el poder de las claves ambientadas asociadas con los efectos reforzantes de las drogas (Aguilar y cols., 2013). Emplearemos una dosis subumbral de cocaína (1mg/kg) la cual es capaz de inducir CPL en animales en condiciones estándar, pero que sin embargo, se muestra efectiva en animales cuyo sistema de refuerzo se ha visto sensibilizado, como sucede tras experiencias de estrés (Montagud-Romero y cols., 2018).

Finalmente, entre las intervenciones anti-inflamatorias estudiadas destacar la administración del fármaco antiinflamatorio indometacina o la administración del neuropéptido oxitocina antes de los episodios de estrés social. Además, se evaluó, si intervenciones basadas en el enriquecimiento ambiental físico e intervenciones sociales, que clásicamente se han mostrado efectivas en el manejo clínico del estrés, ejercen su efecto protector mediante un mecanismo antiinflamatorio.

En cuento a los principales resultados obtenidos, en el primer estudio pudimos validar que el modelo de DS llevado a cabo es un procedimiento valido capaz de inducir en los animales consecuencias a largo plazo como ansiedad e incremento de los efectos reforzantes y motores de la cocaína. Demostramos además la participación necesaria del neuropéptido CRF en estos efectos.

En segundo estudio quedó demostrado el potencial infamatorio tanto a nivel periférico como a nivel central del estrés social, así como la capacidad para inducir un estado de *priming* ante futuros insultos inflamatorios. Igualmente se determinó que el bloqueo de esta respuesta inflamatoria, mediante la administración del antiinflamatorio indometacina, era capaz de revertir el incremento en los efectos reforzantes de la cocaína inducidos por estrés, así como la respuesta inflamatoria.

Similares resultados se obtuvieron en el tercer estudio. El pretratamiento con el neuropéptido oxitocina se mostró efectivo bloqueando la respuesta inflamatoria inducida por estrés, así como la potenciación de los efectos reforzantes y motores de la cocaína evaluados mediante los paradigmas de CPL y auto-administración.

En el cuarto estudio observamos que el enriquecimiento ambiental de tipo social ejercía un efecto protector análogo al observado tras la administración de antiinflamatorios.

Adicionalmente determinamos que esta disminución de los efectos del estrés era dependiente del neuropéptido oxitocina ya que, al administrarse un antagonista de los receptores del mismo, el efecto protector del enriquecimiento social era neutralizado.

En el quinto estudio se evaluaron los efectos de otra intervención ambiental, en este caso del enriquecimiento ambiental. Los resultados muestran que los animales alojados en un ambiente enriquecido desde su llegada hasta el final del estudio presentan una menor reactividad del eje endocrino del estrés. Sin embargo, esta intervención no demostró ningún efecto antiinflamatorio ni protector sobre el CPL. Es más, los animales no estresados alojados en condiciones de enriquecimiento ambiental desarrollaron CPL inducido por una dosis subumbral de cocaína.

Resultados similares se obtuvieron en el sexto estudio donde la intervención ambiental se basó en el incremento de la actividad física de los roedores mediante el acceso a ruedas de actividad. Si bien el resultado específico de cada grupo experimental estuvo condicionado por la modalidad de actividad física a la que se expuso al animal (acceso constante o intermitente) ninguna modalidad de ejercicio mostró los efectos protectores hipotetizados. El acceso a experiencias de ejercicio voluntario en rueda de actividad incrementó los niveles de IL-6 en los animales, así como potenció el establecimiento de CPL de cocaína en comparación a los animales sedentarios. Este efecto se relacionó con el incremento de factores neurotróficos en estos animales en el hipocampo y núcleo accumbens que estaría potenciando los aprendizajes asociativos necesarios para establecer el CPL.

Finalmente, los resultados del séptimo estudio tienen un carácter tentativo y se corresponden a un estudio piloto realizado durante una estancia doctoral en el laboratorio del Dr. Russo. En este experimento se demostró que un protocolo de estrés social crónico era capaz de disminuir la integridad del sistema vascular cerebral, lo que se relacionó con un incremento de la permeabilidad de la barrera hematoencefálica. Igualmente se describió que existía una entrada de células inmunes de la periferia (monocitos) al SNC, que quedaban atrapadas en el espacio perivascular sin llegar a alcanzar el tejido cerebral, ejerciendo desde ese lugar sus efectos inflamatorios.

Globalmente los resultados obtenidos en la presente Tesis nos permiten validar la hipótesis inflamatoria como mecanismo mediador de la vulnerabilidad a los efectos de

las drogas inducida por estrés. El estrés social, en forma de derrota social repetida, posee el potencial de alterar el sistema inmune promoviendo una respuesta inflamatoria aguda así como de inducir un estado de alta reactividad (*priming*). Este *priming* se hace evidente al observar una respuesta exagerada de los animales derrotados tras la administración de cocaína en el CPL. Estos estados proinflamatorios estarían mediando en la vulnerabilidad a padecer consecuencias fisiológicas y conductuales derivadas del estrés. Así lo demuestra el hecho de que el bloqueo farmacológico de la respuesta inflamatoria es capaz de revertir el perfil de ansiedad y el incremento de la respuesta a los efectos reforzantes de la cocaína que caracteriza a los animales derrotados. Igualmente hemos podido demostrar que efectos antiinflamatorios estarían mediando la efectividad de algunas intervenciones clásicas en el manejo de las adiciones, como por ejemplo el apoyo social, que en la presente Tesis hemos modelado como enriquecimiento ambiental social. El alojamiento en parejas se ha mostrado eficaz revirtiendo los efectos inflamatorios del estrés social y también sus efectos potenciadores del refuerzo de la cocaína. Además, hemos podido determinar que estos efectos protectores son dependientes de la liberación de oxitocina, un neuropéptido que promueve el contacto social y que posee efectos antiinflamatorios. Este efecto protector de la oxitocina se ha replicado también cuando se ha administrado exógenamente como un tratamiento farmacológico.

Sin embargo, no todas las intervenciones ambientales estudiadas han tenido el efecto protector hipotetizado. Las dos intervenciones basadas en enriquecimiento ambiental mediante el incremento de la estimulación en el alojamiento y el incremento de la actividad física no han mostrado poseer un efecto antiinflamatorio. Tampoco han sido efectivas contrarrestando los efectos del estrés sobre el establecimiento de CPL, es más, han potenciado el desarrollo de CPL incluso en los animales no estresados. Este efecto estaría promovido por un aumento en los procesos de plasticidad cerebral inducido por el enriquecimiento ambiental lo que permitiría un mejor aprendizaje asociativo en el paradigma del CPL. Por lo tanto, potenciarían el poder de las claves asociadas al consumo de la droga.

La presente Tesis Doctoral ha incrementado el conocimiento sobre las bases neurobiológicas de los efectos del estrés sobre la respuesta a la cocaína, demostrando el papel mediador del sistema inmune. Hemos propuesto además un modelo explicativo

"El círculo vicioso" que integra los efectos del estrés en distintos sistemas cerebrales ayudando a predecir su impacto final en la respuesta a la cocaína. Esperamos que esta investigación ayude a diseñar intervenciones preventivas y tratamientos más efectivos, abriendo la puerta a explorar intervenciones antiinflamatorias en el manejo del estrés y su impacto en la adicción a la cocaína.

# PREFACE

This Doctoral Thesis is based on the following seven studies:

- Ferrer-Pérez, C., Reguilón, M. D., Manzanedo, C., Aguilar, M. A., Miñarro, J., & Rodríguez-Arias, M. (2018). Antagonism of corticotropin-releasing factor CRF1 receptors blocks the enhanced response to cocaine after social stress. *European journal of pharmacology*, 823, 87-95. doi: 10.1016/j.ejphar.2018.01.052 **(Study 1)**

- Ferrer-Pérez, C., Escrivá-Martinez, T., Montagud-Romero, S., Ballestín, R., Reguilón, M. D., Miñarro, J., & Rodríguez-Arias, M. (2018). Indomethacin blocks the increased conditioned rewarding effects of cocaine induced by repeated social defeat. *PloS one, 14*(2), e0212397. doi: 10.1371/journal.pone.0209291 **(Study 2)**

- Ferrer-Pérez, C., Castro-Zavala, A., Luján, M. Á., Filarowska, J., Ballestín, R., Miñarro, J., Valverde, O. & Rodríguez-Arias, M. (2019). Oxytocin prevents the increase of cocaine-related responses produced by social defeat. *Neuropharmacology, 146,* 50-64. doi: 10.1016/j.neuropharm.2018.11.011 **(Study 3)**

- Ferrer-Pérez, C., Reguilón, M. D., Manzanedo, C., Miñarro, J., & Rodríguez-Arias, M. (2019). Social housing conditions modulate the long-term increase in cocaine reward induced by intermittent social defeat. *Frontiers in Behavioral Neuroscience, 13*, 148. doi: 10.3389/fnbeh.2019.00148 **(Study 4)**

- Ferrer-Pérez, C., Reguilón, M. D., Manzanedo, C., Miñarro, J. & Rodríguez-Arias, M. Endogenous oxytocin is essential for the buffering effects of social enrichment against the increase in cocaine reward induced by social stress. *In preparation.* **(Study 4)**

- Ferrer-Pérez, C., Reguilón, M. D., Manzanedo, C., Miñarro, J. & Rodríguez-Arias, M. Environmental enrichment modulates the impact of social stress on the endocrine and immune systems and potentiates cocaine-induced conditioned place preference. *In preparation.* **(Study 5)**

- Ferrer-Pérez, C., Reguilón, M. D., Manzanedo, C., Miñarro, J. & Rodríguez-Arias, M. Effect of voluntary wheel running exercise on the endocrine and inflammatory response to social stress and in the sensitivity to the conditioned rewarding effects of cocaine. *In preparation.* **(Study 6)**

- Ferrer-Pérez., C. Characterization of the neurovascular and immune consequences of chronic social defeat stress and its role in stress-induced depression-like behavior. *Pilot study*. **(Study 7)**

# INDEX

**1. INTRODUCTION** ..... 23

1. General Introduction ..... 25
2. Stress and cocaine addiction ..... 31

2.1.Cocaine addiction ..... 33

2.2.Stress as a risk factor ..... 34

2.3.Neurobiology of stress-induced vulnerability to addiction ..... 36

2.4.Animal models in the study of stress influences in addiction ..... 38

3. Influence of the immune system on drug addiction ..... 41

3.1.Components and function of the immune system ..... 43

3.2.Immune system and mental disorders ..... 45

3.3.Stress modulation of the immune system ..... 48

3.4.Role of inflammation in stress induced vulnerability to drug addiction .... 51

3.5.Anti-inflammatory interventions as a therapeutic approach in stress-related disorders ..... 52

**2. AIMS AND HYPOTHESES** ..... 55

**3. RESULTS** ..... 65

- Study 1-Antagonism of corticotropin-releasing factor CRF1 receptors blocks the enhanced response to cocaine after social stress ..... 67
- Study 2-Indomethacin blocks the increased conditioned rewarding effects of cocaine induced by repeated social defeat ..... 79
- Study 3-Oxytocin prevents the increase of cocaine-related responses produced by social defeat ..... 99
- Study 4-Social housing conditions modulate the long-term increase in cocaine reward induced by intermittent social defeat. ..... 117

-Endogenous oxytocin is essential for the buffering effects of social enrichment against the increase in cocaine reward induced by social stress ..... 129

- Study 5- Environmental enrichment modulates the impact of social stress on the endocrine and immune systems and potentiates cocaine-induced conditioned place preference. ..... 145

- Study 6- Effect of voluntary wheel running exercise on the endocrine and inflammatory response to social stress and in the sensitivity to the conditioned rewarding effects of cocaine ..... 171
- Study 7-Characterization of the neurovascular and immune consequences of chronic social defeat stress and its role in stress-induced depression-like behavior ..... 205

**4. GENERAL CONCLUSIONS** ..... 219

**5. REFERENCES** ..... 231

ABBREVIATIONS

| | |
|---|---|
| ACTH | Adrenocorticotrophic Hormone |
| BBB | Blood Brain Barrier |
| BDNF | Brain-Derived Neurotrophic Factor |
| CNS | Central Nervous System |
| CPP | Conditioned Place Preference |
| CRF | Corticotropin-Releasing Factor |
| DA | Dopamine |
| DAMPs | Damage-Associated Molecular Pattern |
| EE | Environmental Enrichment |
| GCs | Glucocorticoids |
| HPA | Hypothalamic-Pituitary-Adrenal axis |
| IL-1β | Interleukin-1beta |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| LPS | Lipopolysaccharide |
| mRNA | Messenger Ribonucleic Acid |
| NE | Norepinephrine |
| NSAIDs | Non-Steroidal Anti-inflammatory Drugs |
| OT | Oxytocin |
| PAMPs | Pathogen-Associated Molecular Patterns |
| POMC | Pro-opiomelanocortin |
| SAM | Sympathetic-Adrenal Medullary system |
| SD | Social Defeat |
| SUD | Substance Use Disorders |
| TLR | Toll-like Receptor |
| TNF- α | Tumor Necrosis Factor-alpha |
| TNF-β | Tumor Necrosis Factor-beta |
| VTA | Ventral Tegmental Area |
| VWR | Voluntary Wheel Running |

# 1. INTRODUCTION

# 1. General Introduction

## 1. General introduction

Stress response is a healthy and adaptive strategy that helps us to cope with situations of acute challenge to our survival (Ulrich-Lai & Herman, 2009). However, in our modern society, stress has become a significant problem. We live in a complex social environment with constant demanding conditions that are inescapable parts of our daily routine. We face persistent "quotidian" stressors (Pearlin & Skaff, 1995), mainly everyday concerns such as role conflicts between work and family, arguments with children or unexpected deadlines (Almeida, 2005) that can affect our welfare more than major but less frequent life events (Serido et al., 2004).

This exposure to chronic stress may promote long-lasting adverse effects on our health, both in physiological and psychological dimensions (Hartsell & Neupert, 2017; Lupien, et al., 2009; McEwen, 2004). In fact, there is vast literature linking stress to some epidemic diseases in our society, such as cancer (Krizanova et al., 2016), obesity and depression (Ouakinin et al., 2018). In addition, new studies in the field of addiction have highlighted the role of stress in all the stages of the addictive process, promoting the initiation, maintenance, escalation of intake and relapse into drug taking (Koob, 2008; Koob & Schulkin, 2018).

Within the neuroscientific perspective, drug addiction can be considered a multifactorial disorder of chronic relapse as a result of the interaction between biological and environmental factors that lead to the loss of control over the use of the drug (Kalivas & Volkow, 2005). Stressful events have been considered a risk factor that interplay with biological factors, promoting enhanced vulnerability to substance use disorders (SUD) (Ruisoto & Contador, 2019). Moreover, some stages of addiction, such as the negative state experienced during withdrawal or while facing negative consequences from drug consumption, can trigger physiological and psychological stress responses by themselves (Koob & Schulkin, 2018), which in turn may complete the loop of drug addiction and stress.

Knowing that drug addiction has a profound impact in all the domains of human functioning, as it compromises interpersonal, economic and health status (Birkeland et al., 2018), it is necessary to develop effective strategies to manage the consequences of

stress over the addictive process. To that end, it is first essential to identify what the main source for our daily stress is. Despite the fact that physical stressors can be highly threating, the main source of human stress stems from social interactions tensions (Dickerson & Kemeny, 2004; Tamashiro et al., 2005). Although positive social interactions can act as natural reinforcers with a protective effect, social stress experiences, such as isolation or bullying in the workplace, are linked to higher rates of drug abuse and more vulnerability to relapse after periods of detoxification (Heilig et al., 2016; Niedhammer et al., 2010; Sullivan et al., 2006). Consequently, there is growing interest to unravel how social factors modulate individual drug response (Neisewander et al., 2012). However, there is currently no complete characterization of the neurobiological mechanisms involved in the effects of social stress (Ruisoto & Contador, 2019).

One interesting approach to study social stress-induced vulnerability to mental disorders is to use animal models. Within all the paradigms that model social stress in rodents, social defeat (SD) is considered the most representative (Hammels et al., 2015; Neisewander et al., 2012), as it mimics the reality of subordinate vs. aggressor relations in humans (Björkqvist, 2001; Selten et al., 2013). Also named the resident-intruder paradigm, it is based on the territorial attack of a resident male to a conspecific intruder, allowing basic researchers to study the short- and long-term behavioural and physiological consequences of social stress (see revision in Montagud-Romero et al., 2018). Overall, the scientific literature confirms that social stress experiences using SD protocols enhance rewarding responses to drugs and precipitate the reinstatement of drug seeking in experimental paradigms such as the conditioned place preference (CPP) (Montagud-Romero et al., 2018) and the self-administration (Rodríguez-Arias et al., 2016, 2017) paradigms, where these effects persist even one month after the last social defeat.

Several neurobiological theories have emerged to explain the mechanism that leads to the abovementioned stress-induced vulnerability, for example, alterations in the corticotrophin-releasing factor (CRF) system (Zorrilla et al., 2014), dopamine (DA) neurotransmission system (Reguilón et al., 2017) or epigenetic forms of plasticity (Montagud-Romero et al., 2016). Recent basic research has pointed to a new target: the immune system. Evidence shows that social stress induces the activation of the immune

system in the form of an inflammatory state, both in the periphery, but also in the central nervous system (CNS) (Hodes et al., 2014; Pfau & Russo, 2016). This stress-induced immune activation is being posited as a link between stress and disease through the alteration of neuroendocrine functions.

This link between social stress-induced immune activation and mental illness has already been well characterized in animal models of anxiety and depression (Hodes et al., 2016; Ménard et al., 2017). It is also known that SUDs are related to changes in the immune system activity (Clark, et al., 2013; Cui et al., 2014) and neuroinflammatory processes could help to explain some of the effects of drugs, including toxicity, deleterious cognitive effects and reward (Coller & Hutchinson, 2012; Hutchinson & Watkins, 2014; Montesinos et al., 2014; 2016).

Rather than owing to a single mechanism, we theorize that the consequences of social stress in SUDs are the result of the interplay between multiple and complex mechanisms in the peripheral systems and the CNS. We hypothesize that within all these physiological changes, the immunological response to stress is a key mechanism to understand how social stress potentiates the effects of drugs. To this end, the first objective of the present work was to determine whether social stress was able to trigger an immunological response in our experimental animals. Then, after the confirmation of the inflammatory potential of social stress, we aimed to determine whether this inflammatory response was in fact contributing to the long-lasting effects of social stress. To reach this goal, we explored if some anti-inflammatory interventions (pharmacological and behavioural) were effective in preventing the inflammatory response activated by social stress and the long-term potentiation of the effects of drug abuse.

# 2. Stress and cocaine addiction

### 2.1. Cocaine addiction

Cocaine is a powerful stimulant of the CNS obtained from a natural alkaloid that is extracted from the Erythroxylon coca plant (Cregler, 1989; Zimmerman, 2012). This plant originates in South America and its use by Indians of the Andes to inhibit hunger, thirst and fatigue dates back to 600 A.D. (Biondich & Joslin; 2016). Coca leaves are soaked in organic solvents to form a paste that, after the addition of hydrochloric acid, results in the formation of cocaine hydrochloride salt. The obtained compound is then soluble to water and can be injected, ingested or snorted intranasally. Additionally, cocaine can be also smoked by hardening it to a rock state with the addition of a base (Zimmerman, 2012). In Europe, cocaine is the second most widely used illicit drug after cannabis, and the preferred psychostimulant in Spain (EMCDDA, 2018). Last year, prevalence of cocaine use among the Spanish population was estimated at 2%, with consumption being higher in men than women (3.2% vs. 0.8%) (EDADES, 2017).

Cocaine has an excitatory effect in the CNS and produces pleasure through its actions in the mesolimbic DA system, known as the reward pathway, which is naturally activated by stimuli such as food or sex. What this drug does is to interfere with normal communication between neurons in this reward circuit, enhancing the levels of DA by blocking its reuptake (Nestler, 2005). The subjective experience of a cocaine dose is euphoria, excitement, increased alertness and self-esteem as well as a general sense of well-being (Cregler, 1989). The fast onset of these rewarding effects, which appear within 5 minutes after intranasal use, 10-60 seconds after intravenous injection and only 3-5 seconds after being inhaled (Zimmerman, 2012), confers cocaine a perfect profile to trigger a compulsive consumption that can lead to cocaine use disorders and addiction (Cadet et al., 2014).

Addiction is the most severe form of cocaine use disorders (NIDA, 2018) and can appear after repeated exposure to the substance. With regular use of the drug, a tolerance to its pleasant effects may appear, and higher and more frequent cocaine dosage would be needed to produce the same level of reward (Buttner, 2012). Simultaneously, users also develop a sensitization to some negative effects, such as anxiety, convulsion or toxic consequences (Riezzo et al., 2012). These effects are related to neuroadaptations that appear after a long exposure to the drug, mainly in two

brain pathways: the reward system and the stress circuits (Buttner, 2012). The reward pathway becomes less sensitive to natural reinforcers while the stress circuits become overreactive, promoting states of displeasure and negative mood that can be relieved with repeated drug administration. As a consequence, during withdrawal, drug users experience an inability to control the impulse to take the drug again rather than search for natural rewards such as social interactions, food or exercise (Volkow et al., 2012).

Addiction can be defined as a chronic brain disease, characterized by compulsive drug seeking and relapse (Cadet et al., 2014). This compulsive use of the drug continues despite its negative impact in all personal spheres. Reports show that chronic cocaine users suffer from severe organic complications that can be life-threating, such as vascular damage within the brain, myocardial infarction, cerebral atrophy, premature aging (Ersche et al., 2013; Siniscalchi et al., 2015), or psychiatric disorders such as depression and psychosis (Karila et al., 2012). On the socioeconomic field, problems such as housing instability, homelessness, criminal behaviours, unemployment or dependence on welfare are common among drug addicts (Daley, 2013).

From all of these devastating consequences arises the necessity to develop preventive strategies against the initiation of drug consumption and to develop effective therapeutic interventions for patients suffering from addiction. The present Doctoral Thesis is focused on the consecution of these two objectives. On the one hand, we aimed to detect and define the risk factors that make subjects more vulnerable to develop cocaine addiction, this information being useful to guide new preventive approaches. On the other hand, we are also oriented in the research of pharmacological and behavioural therapeutic targets, which would lead to novel interventions in the treatment of cocaine addiction.

### 2.2. Stress as a risk factor

Individual life experiences modulate the response to drugs of abuse. Specifically, adverse life experiences can render individuals more prone to suffering from substance use disorders and make them more vulnerable to relapse after periods of detoxification (Koob & Schulkin, 2018; Sinha et al., 2011). Stress is an adaptive response of the organism to overcome physiological and psychological demanding situations that threaten the homeostasis maintenance and, eventually, survival (Jones et al., 2016;

Krizanova, et al., 2016). To handle this challenging situation, the organism displays a general stress response consisting of the activation and deactivation of several systems of regulation (Selye, 1975). Initially, these changes are non-specific and promote a fast mobilization or provide resources to face the threat while inhibiting other biological functions that are not immediately necessary for survival. The physiological response comprises an increase in the tone of the cardiovascular and respiratory systems and changes in metabolism and energy functions; and on the behavioural sphere, there is an increase in arousal, vigilance and alertness (Tsigos et al., 2016). Together, these responses allow the organism to increase the resources available to handle the stressful situation.

Negative consequences of stress appear when challenges are persistent and there is a prolonged activation of this stress response. The chronic over-activation of several regulatory systems such as the hypothalamic-pituitary-adrenal axis (HPA), together with the chronic inhibition of others such as the reproductive system, result in a "disease of adaptation" (Selye, 1975) that has a deep negative impact in the organism (Tsigos et al., 2016). In fact, in our society, people usually experience a highly stressful lifestyle coupled with constant demanding situations related to finance, work and social relations. In this context, severe and prolonged stress exposure is translated into an increased risk of suffering from physical and psychological disorders or stress-related disorders (Liu et al., 2017).

The most common stress-related diseases are cardiovascular problems such as hypertension, metabolic diseases such as diabetes or obesity, and even cancers caused by the inhibition of the immune system (Krizanova, et al., 2016). Stress also has negative psychological consequences, promoting the appearance of disorders such as depression, anxiety and sleep problems, among others (Cohen et al., 2007; Liu et al., 2017). Regarding our focus of interest, stress is also considered a risk factor with a negative influence in all stages of addiction. It has a key role in promoting the initiation, maintenance and escalation of drug intake, as well as in its chronicity when promoting relapse (Montagud-Romero et al., 2018; Sinha et al., 2011). Epidemiological studies show that early life stress exposures increase the prevalence of mental disorders, including reward dysfunctions such as drug addiction (Hyman, et al., 2007; Thatcher et al., 2008). During adulthood, negative social experiences such as isolation or bullying in

the workplace are also linked to higher rates of drug abuse and more vulnerability to relapse after periods of detoxification (Niedhammer et al., 2010; Sullivan et al., 2006).

### 2.3. Neurobiology of stress-induced vulnerability to addiction

The central structures of control of the neuroendocrine stress response are the HPA axis and the sympathetic-adrenal medullary (SAM) axis. In response to stress, the HPA axis releases CRF from the paraventricular nucleus of the hypothalamus (Arnett et al., 2016). This hormone is then released into the portal circulation system, which connects the hypothalamus and the adenohypophysis. In parallel, CRF axons project to extrahypothalamic areas such as the extended amygdala and ventral tegmental area (VTA) (Haass-Koffler & Bartlett, 2012). When CRF binds to its receptors located in the adenohypophysis, it promotes the synthesis of pro-opiomelanocortin (POMC), a polypeptide precursor of smaller peptides, such as the adrenocorticotrophic hormone (ACTH), α-melanocyte-stimulating hormone and β-endorphin (Arnett et al., 2016). After being synthesized, ACTH is released into systemic circulation where it can interact with the adrenal cortex, inducing the synthesis of glucocorticoids (GCs). Cortisol in humans and corticosterone in rodents are the final hormonal effectors of the HPA axis (Tsigos et al., 2016). GCs receptors are ubiquitously expressed in the organism and support the general stress response. On one hand, GCs receptors promote catabolic reactions with the aim of obtaining energy to overcome the stressful situation, and on the other hand, they supress functions that are not immediately necessary for survival with the objective of optimizing resources having anti-growth, anti-reproductive and immunosuppressive effects (Goeders & Clampitt, 2002). A negative feedback loop regulates the optimal function of this system, preventing excessive release of GCs. The detection of GCs promotes the termination of the stress response, the decrease of production of CRF at the hypothalamic level, and the decrease of POMC synthesis in the adenohypophysis (De Kloet, et al., 2005; Tsigos et al., 2016).

On another note, there is an activation at the hypothalamic level of the sympathetic branch of the autonomic nervous system that stimulates the release of catecholamines by the adrenal medulla (Tsigos et al., 2016). The release of catecholamines epinephrine and norepinephrine (NE) promote the increase in heart rate and blood pressure, the conversion of glycogen into glucose by the liver, and a shift in blood flow to the skeletal

muscles (Goeders, 2003). For more detailed information about neuroendocrine response to stress, see Figure 1.

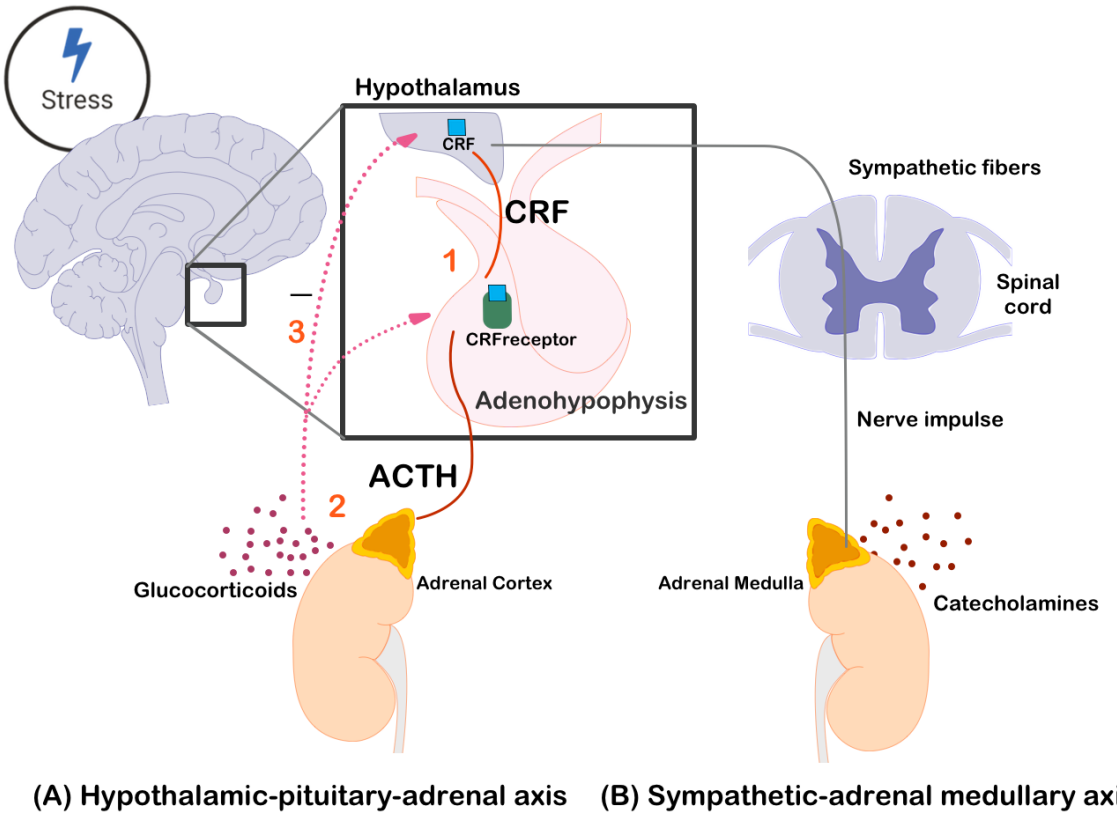


**Figure 1. Schematic representation of the hypothalamic-pituitary-adrenal axis and the sympathetic-adrenal medullary axis in the neuroendocrine response to stress.**

(A) **1.** Hypothalamic-pituitary-adrenal (HPA) axis. Stress signals trigger the release of corticotropin-releasing factor (CRF) from a subset of neurons in the paraventricular nucleus of the hypothalamus. **2.** CRF is transported to the adenohypophysis, where it binds to CRF receptors stimulating the synthesis and release the adrenocorticotrophic hormone (ACTH) into the general circulation. In the adrenal cortex, ACTH binds to the melanocortin type 2 receptors and stimulates the secretion of cortisol (glucocorticoids in rodents) into the general circulation. **3.** Cortisol inhibits HPA axis activation (negative feedback) at the hypothalamic and pituitary level. (B) Stress signals stimulate the sympathetic branch of the autonomic nervous system. Pre-ganglionic neurons release acetylcholine that stimulates post-ganglionic neurons to release norepinephrine into the adrenal medulla that eventually releases the catecholamines epinephrine and norepinephrine.

There is an enormous overlap between the neurobiological substrates that are involved in stress response and the those involved in drug addiction (Koob & Schulkin, 2018).

In the VTA, a key structure of the reward pathway, there are dopaminergic neurons that express receptors sensitive to CRF (Boyson et al., 2014; Haass-Koffler & Bartlett, 2012). CRF in this area increases the activity of dopaminergic neurons, which are projecting to other areas of the reward pathway such as the nucleus accumbens, the amygdala and prefrontal areas (Steketee & Kalivas, 2011). This hyperactivity of the DA system within the mesolimbic pathway would translate into an increased sensitivity in rewarding and motor properties of different substances of abuse, including cocaine (Han et al., 2017; Sinha et al., 2011). In addition, increased levels of DA induced by CRF in other structures such as the extended amygdala are especially relevant during withdrawal (Zorrilla et al., 2014). The overactivation of this structure has a key role in the negative emotional state and anxiety-like effects experienced during abstinence and helps to explain the negative reinforcement processes that drive the compulsivity of addiction (Zorrilla et al., 2014).

### 2.4. Animal models in the study of stress influences in addiction

Animal models are a useful to increase the understanding about the neurobiological mechanisms underlying the effects of stress on drug addiction. As previously commented, social experiences are powerful determinants of behavior and health status central to human development (Ajonijebu et al., 2017). In this regard, within all the paradigms that model social stress in rodents -such as social deprivation, social instability, territorial and maternal aggression-, social defeat (SD) is considered the most representative for studying the physiological and behavioral consequences of social stress (Hammels et al., 2015; Neisewander et al., 2012). In fact, this stress model has been proven to produce severe stress responses that translate into dramatic increases in GCs levels (Koolhaas et al., 2011). SD is a useful tool to recreate experiences such as bullying, physical abuse or subordination (Miczek et al., 2008) as this paradigm closely mimics the reality of subordinate vs. aggressor relations in humans (Björkqvist, 2001). Also named the resident-intruder paradigm, it is based on the territorial attack of a resident male confronted with a conspecific intruder. In these agonistic encounters, resident and intruder animals demonstrate natural offensive and defensive behaviors, which allows researchers to study the short- and long-term behavioral and physiological consequences of social subjugation stress (Shimamoto, 2018).

After being exposed to repeated episodes of SD, rodents display depression-like symptoms such as anhedonia, heightened anxiety, social withdrawal and impaired cognitive functions (Higashida et al., 2018). Regarding its impact in drug-related behaviors, the scientific literature points to exposure to different procedures of SD as increasing the rewarding and reinstating effects of psychostimulant drugs, such as cocaine, using the self-administration and CPP paradigms (Burke & Miczek, 2015; Han et al., 2017; Montagud-Romero et al., 2018; Neisewander et al., 2012; Reguilón et al., 2017; Shimamoto, 2018). Socially stressed animals also show an enhanced sensitivity to cocaine-induced hyperactivity and dopaminergic cross-sensitization, with an augmented behavioral response to subsequent doses of cocaine after repeated drug exposure (Covington, et al., 2008). A detailed description of the protocols of SD and CPP mentioned in this section will be exhaustively explained in the body of the publications of the present Doctoral Thesis.

To sum up, SD 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. Moreover, SD stress can act as a precipitant factor in the reinstatement of drug seeking in the self-administration and CPP paradigms. A full review of this topic can be found in the review "Social defeat stress: Mechanisms underlying the increase in rewarding effects of drugs of abuse" (Montagud-Romero et al., 2018), in which the author of the present thesis has participated.

# 3. Influence of the immune system on drug addiction

### 3.1. Components and function of the immune system

Our immune system encompasses a group of defence mechanisms, structures and biological processes that are triggered against aggressions and threats to our organism homeostasis. These aggressions can come from the external environment, as in bacteria, viruses, fungi, and parasites, or from the internal environment, as in cancer or damaged cells. For this purpose, many organs, cells and molecular pathways work together in a global immune system (Masi et al., 2017), where two big sub-systems of immunity response can be distinguished: the innate (non-specific) and acquired (adaptive) systems (Mackay et al., 2000). The innate immune system is the first-line of defence against pathogens. It consists of all the defensive strategies that do not have an immunology memory, and thus they do not change even if previous encounters with the pathogen had taken place (Chaplin, 2010; Nicholson, et al., 2016). The innate immune system is composed of physical barriers, such as the epithelial layers of the skin, mucus in the body cavities, as well as chemical barriers such as enzymes and acidic pH (Chaplin, 2010). When any pathogen breaks through these initial barriers, the cellular innate immune response will be activated. Pathogens have characteristic pathogen-associated molecular patterns (PAMPs) of carbohydrates, such as mannose, which are uncommon in the cells of vertebrates (Fraser et al., 1998, 2004; Mogensen, 2009). Innate immune cells such as macrophages, dendritic cells and neutrophils have receptors in their surface that can recognize PAMPs and discriminate between these "non-self" molecules and our "self" molecules. Additionally, innate immune cells can also recognize damage-associated molecular patterns (DAMPs) released by "self" damaged or dying cells (Roh & Sohn, 2018). The detection of PAMPs and DAMPs by the pattern-recognition receptors of immune cells can stimulate macrophages and neutrophils to engage in the process of phagocytosis and degradation of the pathogen that will lead to the destruction of the threat. Other consequence of the activation of pattern-recognition receptors can be the release of chemical substances by immune cells such as antimicrobial compounds or chemoattractant substances as cytokines and chemokines (Mackay et al., 2000). Cytokines are recruiting proteins, such as tumor necrosis factor-α (TNF-α), Interleukin-1ß (IL-1ß) and Interleukin-6 (IL-6), that attract cells, molecules, and fluid into the affected site, causing inflammation (Chaplin, 2010; Roh & Sohn, 2018).

When pathogens cannot be neutralized by the innate immune system, the acquired immune responses will be activated. Dendritic cells can stimulate cells from the acquired immune system by presenting them as antigens (Yatim & Lakkis, 2015). This response of the acquired immune system is more antigen-specific than the innate response, as it comprises mechanisms capable of recognising and eliminating previously known pathogens (Mackay et al., 2000). This purpose is carried out by lymphocytes through two different strategies: one is dependent on B lymphocytes (B cells), which secrete antibodies (immunoglobulins) that neutralize extracellular microorganisms after being stimulated; the other is dependent on T lymphocytes (T cells), which help B lymphocytes to produce antibodies and activate macrophages to kill pathogens (Fraser et al., 2004). For a visual scheme of the components of innate and acquired immunity, see Figure 2.

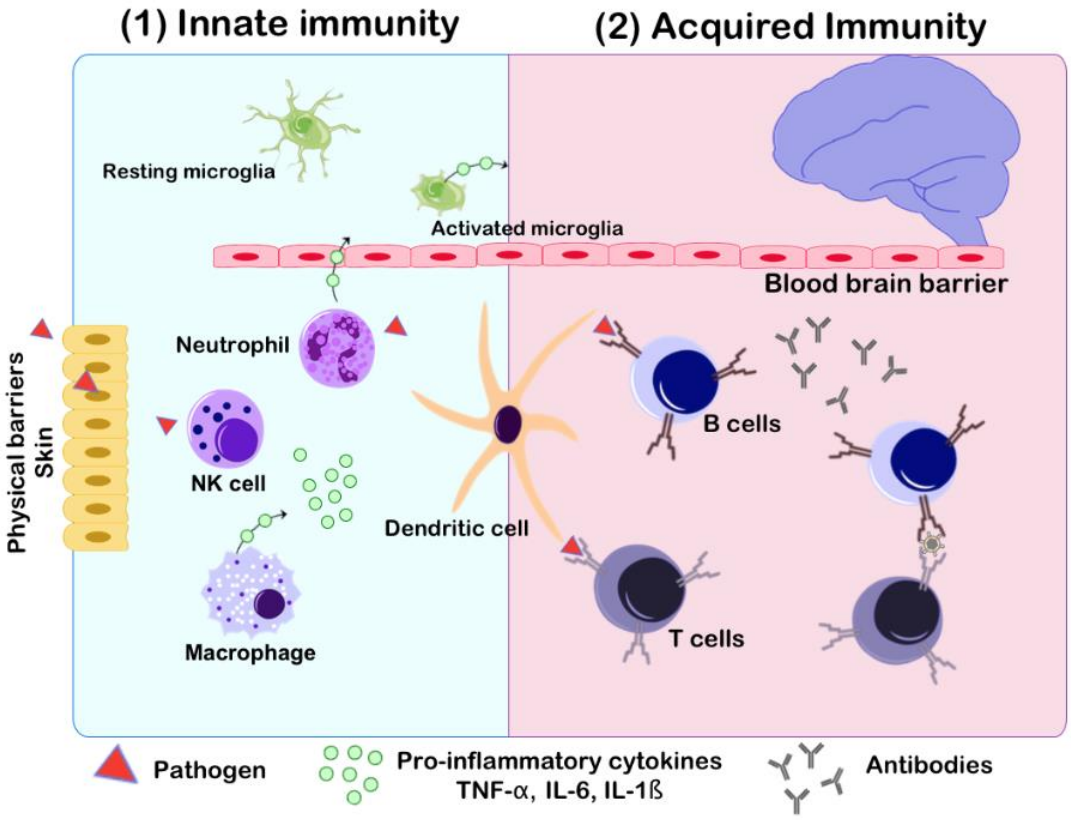


**Figure 2. Components of the innate and acquired immune system**

(1) The innate immune system. If pathogens break through initial physical barriers, the cellular innate immune response will be activated. Innate immune cells are natural killer (NK) and phagocytic cells (macrophages and neutrophils) that have receptors that recognize pathogens and self "damaged" or infected cells. Once activated, they engage

in processes aimed at destroying the pathogen, such as phagocytosis, as well as releasing cytokines and chemokines that will attract cells and fluid into the affected site, causing inflammation. Eventually, cytokines can enter into the brain through the blood brain barrier, activating resident immune cells (microglia). (2) Acquired immune system. When pathogens cannot be neutralized, dendritic cells can stimulate acquired immune system cells by presenting them as antigens. When activated, T cells and B cells secrete antibodies that neutralize extracellular microorganisms. The generation of cell memory in T and B cells results in a specific and faster immune response upon recurrent infection.

The relatively independent functioning of the immunity within the CNS should also be remarked. Traditionally, the brain has been considered an immune, privileged organ protected by the blood brain barrier (BBB) that acts as an interface between the CNS and the systemic circulation (Engelhardt et al., 2017). Under homeostatic conditions, BBB prevents pathogen invasion and, additionally, blocks alarm signals from the peripheral immune system, restricting the entry of immune cells and the diffusion of cytokines (Banks et al., 2005). Thus, immune defence and tissue homeostasis within the CNS is carried out by brain-resident cells, such as microglia (Andreasson et al., 2016).

Innate and acquired immune responses work together as a regulated and global immune system. The final aim of this system is to protect the organism against a universe of external pathogens and against self-threats that compromise the normal functioning of the body (Masi et al., 2017). A deregulated immune system will compromise not only host defence capacities, but also global organism functioning.

### 3.2. Immune system and mental disorders

The genesis of multiple diseases has been directly related to an aberrant function of the immune system (Frank et al., 2016; López & Bermejo, 2017; Soria et al., 2018). Some disorders are caused by a hyperactivity of this system, such as the observed in food allergy, where immune systems react against proteins of several foods (Boyce et al., 2011), or as is the case with autoimmune disorders, where there is a misidentification of "self" cells as "foreign" cells (Davidson & Diamond, 2001). Moreover, over the past few decades, there has also been growing evidence for a link between immune system deregulation and a number of mental illnesses (Chistyakov et al., 2018; Dantzer, 2017).

There is a bidirectional interaction between the nervous and the immune systems (Masi et al., 2017), since each one can influence the function of the other (Filiano et al., 2015). The CNS can influence immune signalling through vagal innervation and neurohormonal axes, such as the HPA and SAM through the release of catecholamines and GCs (Dantzer, 2017; Frank et al., 2013; Soria et al., 2018). On the other hand, the CNS can receive and process signals from the immune system (Lasselin et al., 2018). A clear demonstration of this is the altered behaviour that appears during an infection, which is characterized by social avoidance, anhedonia and lethargy. These symptoms are components of an adaptive response to save energy and promote survival during infection or wound healing, since defensive and restorative processes, such as fever response, have extreme metabolic costs (Dantzer, 2017; Miller & Raison, 2016). The signals that mediate the appearance of this sickness behaviour are the aforementioned pro-inflammatory cytokines, such as TNF-α, IL-1ß and IL-6, released by peripheral immune cells (Dantzer et al., 2008). These soluble molecules have been proven to have a direct impact on neuronal function, and even more, they can activate resident immune cells, turning microglia into a pro-inflammatory state known as neuroinflammation (Pascual et al., 2012; Soria et al., 2018). As previously discussed, cytokines are relatively large molecules that cannot easily diffuse into the brain, but intense immune challenges can compromise the integrity of the BBB, increasing its permeability (Rodríguez-Arias et al., 2017). As a consequence, cytokines and even immune cells can traffic into the brain through the leakiest regions of the BBB as in circumventricular areas (Cathomas et al., 2019; Ménard et al., 2017b).

From the observation that there was a concordance between the symptoms observed in sickness behaviour and the ones observed in depression emerged the theory of neuroinflammation in the genesis of depression. Several clinical studies show that patients diagnosed with major depression have increased levels of circulating pro-inflammatory cytokines such as IL-1ß, IL-6, Interleukin-8 (IL-8), Interleukin-12 (IL-12), interferon-y and TNF-α (Hodes et al., 2014; O'Brien et al., 2007). There is evidence supporting that this elevation in peripheral inflammatory markers predates the occurrence of developing a major depressive disorder, since patients who suffer from chronic inflammatory conditions such as rheumatoid arthritis have an increased risk of suffering from mood disorders such as depression or bipolar disorder (Leboyer et al., 2012). Moreover, pharmacological increases in pro-inflammatory markers, as is the case

with the treatment of hepatitis C with interferon, a potent inducer of cytokines, provoke relevant depressive symptomatology in 25% of patients (Udina et al., 2012). Lastly, measurements of inflammatory activity within the CNS using positron emission tomography have shown that microglia pro-inflammatory activity positively correlated with the severity of depression (Setiawan et al., 2015).

The role of inflammatory conditions to the risk of mood disorders has also been replicated in animal models. The injection of lipopolysaccharide (LPS) from bacterial endotoxin induces a strong inflammatory response that causes depressive-like behaviour in rodents with decreased preference for sucrose and social avoidance, which can be dumped using antidepressant drugs (Ohgi et al., 2013). This depressive status can be induced directly via the injection of pro-inflammatory cytokines such as TNF-α (Dantzer et al., 2008).

Besides mood disorders, it is widely accepted that alterations in inflammatory parameters are linked to the vulnerability to different mental illnesses such as autism, schizophrenia and even SUD (Clark et al., 2013; Cui et al., 2014; Fiedorowicz et al., 2015; Ménard et al., 2017b; Teixeira et al., 2008).

In this regard, several substances of abuse have been reported to induce adaptations in the immune system (Harricharan et al., 2017; Nennig & Schank, 2017). The inflammatory potential of ethanol is well established. Ethanol can interact with Toll-like receptors (TLR) (see Figure 3), which are pattern recognition receptors expressed in immune cells (Montesinos et al., 2015, 2016) and stimulate the production of inflammatory markers within the brain via the activation of microglia and astrocytes (Guerri & Pascual, 2018). The constant state of brain inflammation in alcoholics is harmful, causing myelin reduction, atrophy of the dendrites, abnormal neurogenesis and neurodegeneration (Alfonso-Loeches et al., 2010), which correlates with cognitive deficits (Montesinos et al., 2015). Morphine is another depressant drug that has been proven to induce an inflammatory state. Chronic administration of morphine increases circulating pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, and activate glial cell immunological responses in rodents (Chen et al., 2012, Kohno et al., 2019; Raghavendra et al., 2003).

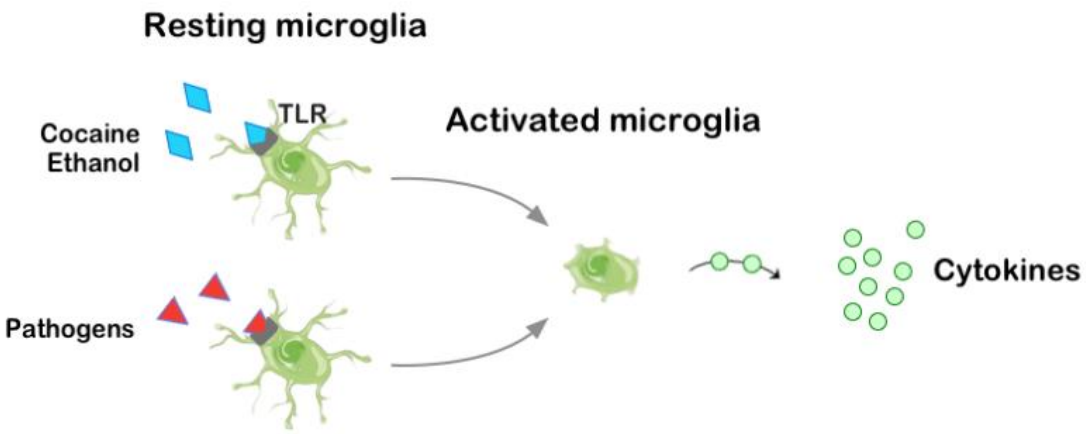


**Figure 3. Ethanol and cocaine promote microglial activation and cytokines release through their interaction with Toll-like receptors (TLR)**

Regarding psychostimulants, both preclinical and clinical studies have shown that drugs such as cocaine and methamphetamine promote the activation of the microglia (see Figure 3) and peripheral immune cells (Moratalla et al., 2017; Sil et al., 2018; Xu et al., 2017). Cocaine users have an increased ratio of pro-inflammatory to anti-inflammatory markers with enhanced plasmatic levels of the pro-inflammatory cytokine IL-6 and a reduction of circulating levels of the anti-inflammatory factor IL-10 (Moreira et al., 2016). Additionally, repeated cocaine use increases the permeability of the BBB, thereby enhancing the entry of peripheral cells into the CNS (Kousik et al., 2012; Rodríguez-Arias et al., 2017), and promotes oxidative stress with an increased reactive oxygen species production (López-Pedrajas et al., 2015), amplifying even further the inflammatory consequences of cocaine consumption and its associated neuropathological dysfunctions.

### 3.3. Stress modulation of the immune system

Recently, inflammatory processes have emerged as a new biological mechanism in the interplay between stress and stress-related diseases (Rohleder, 2014). The neuro-hormonal response to stressors provokes a deep impact in the immune response (Padgett et al., 2003). The activation of the HPA axis and the SAM, along with their respective neuroendocrine cascades, has as its final response the release of GCs and catecholamines (epinephrine and NE). Cells from the innate and the acquired immune systems, such as macrophages and lymphocytes, express receptors that are sensitive to the neuroendocrine products of the HPA and SAM axis. There is solid evidence

showing that GCs have anti-inflammatory and immunosuppressive properties as they inhibit lymphocyte proliferation and cytotoxicity (Padgett et al., 2003). Moreover, in-vitro studies show that immune cells treated with GCs produce less pro-inflammatory cytokines such as TNF-α and enhance the expression of anti-inflammatory cytokines such as IL-10 and TNF-ß (Sorrells et al., 2009).

Thus, a paradox emerges: stress related disorders such as depression correlate with increased levels of pro-inflammatory cytokines, but stress promotes the release of glucocorticoids that have anti-inflammatory actions. This paradox can be solved by focusing on the temporal course of the stress response. During the acute response, GCs display immunosuppressive effects, but during the phase of recuperation, GCs can act as an alert signal for innate immune cells against future inflammatory threats (Frank et al., 2013). After neutralizing a pathogen, the immune system recuperation response does not simply return to baseline levels, but instead, immune defences stay vigilant, and GCs seem to be mediating this primed state, acting as a neuroendocrine warning signal (Frank et al., 2013). For instance, the exposure of a physical stressor such as tail-shock enhances the peripheral and the central inflammatory response to an immune challenge (LPS) applied 24 hours after the stress experience (Johnson et al., 2005). This enhancement of the immune response induced by stress can be pharmacologically blocked by neutralizing GCs signalling (Munhoz et al., 2006).

In the CNS, the exposition to stressors induce fast and brief releases of cytokines in stress-reactive areas such as the hypothalamus and the hippocampus (Johnson et al., 2005). Stress exposure activates resident microglia changing its immunophenotype into a pro-inflammatory state (Frank et al., 2016; 2019). In fact, in our research group, social stress experiences during adolescence were found to induce a long-lasting activation of microglia that persists until adulthood and that modulates the response to further inflammatory challenges, such as the administration of cocaine (Rodríguez-Arias et al., 2018).

Activated microglia eventually induce a neuroinflammatory state accompanied by sickness behaviour, similar to the produced state after a peripheral immune challenge. The mechanism underlying this fast increase in pro-inflammatory signalling in response to stressors is caused by a direct action of NE (Johnson et al., 2008). The activation of adrenergic receptors has been proven to increase the IL-1 mRNA expression in resident

immune cells (Johnson et al., 2005), while the administration of beta-adrenergic receptor antagonists before stressful episodes prevents the increase in central cytokines and the respective psychological and behavioural consequences (Wohleb et al., 2011). In fact, the fast responsivity of microglia after stress makes some researchers postulate that these cells are immunosensors that rapidly respond to alterations in homeostasis and integrate the neural response to threats (Frank et al., 2019).

Lastly, this relation between stress axis and the immune system is not unidirectional. Increased levels of circulating pro-inflammatory cytokines such as IL-6 and TNF-α stimulate the HPA axis and promote the release of CRF and eventually GCs (Silverman et al., 2004). These GCs, in turn, act as a negative feedback control and inhibit the production of pro-inflammatory factors, promoting the resolution of inflammation (Tracey, 2009). For a graphical representation of the process, see Figure 4.

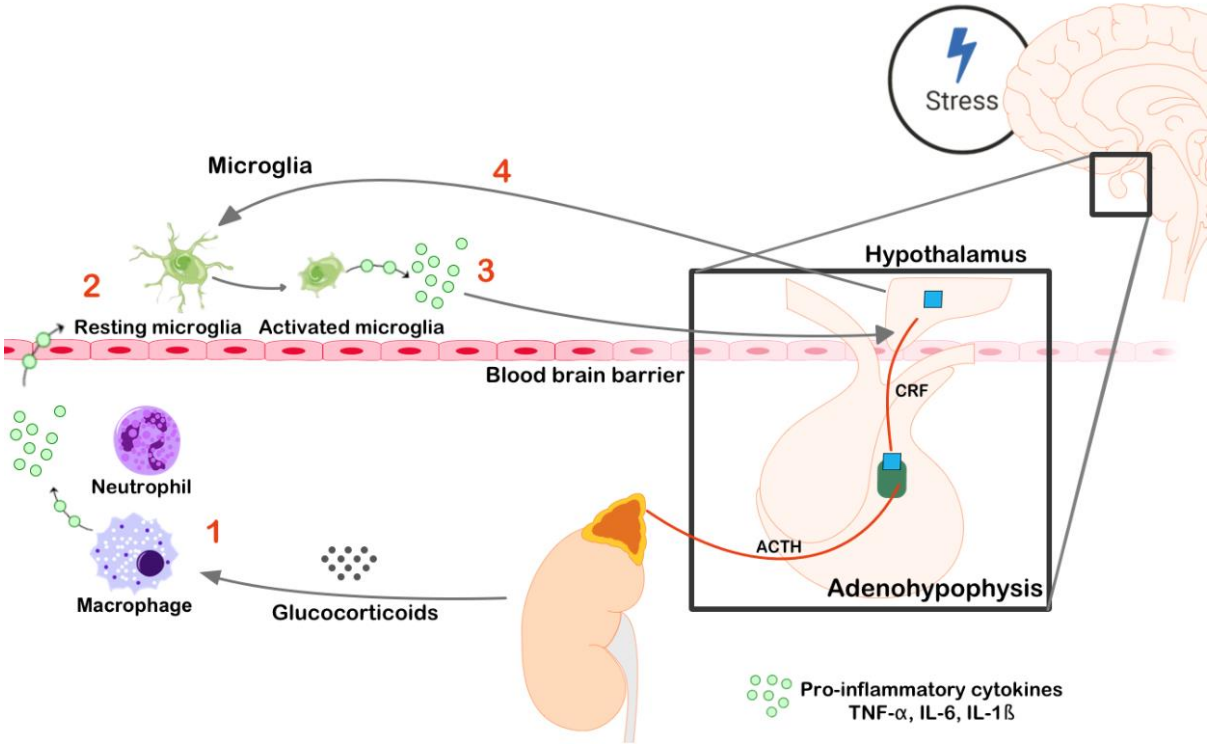


**Figure 4. Interplay between the HPA axis and the innate immune system**

**(1)** Recurrent release of glucocorticoids after repeated stress will turn peripheral immune cells into a primed state with enhanced response to further immune challenges. **(2)** The activation of stress-primed immune cells would induce an exaggerated pro-inflammatory response that can eventually reach the brain, promoting the activation of resident immune cells. **(3)** Activated microglia would release pro-inflammatory cytokines that activate the HPA axis and promote the release of CRF. **(4)** Stress-induced

norepinephrine signalling can directly activate microglia, turning these immune cells into a pro-inflammatory state.

Periods of intense stress can over-activate the HPA axis leading to deregulation of this feedback loop and causing an imbalance between its inflammatory and anti-inflammatory role while shifting it into a reactive state with enhanced immune responses (Ambrée et al., 2018; Avitsur et al., 2009: Frank et al., 2013). As a consequence, individuals with a history of negative adverse experiences (stressors) have a hyper activated stress axis that facilitate exaggerated immune responses, even against minimal inflammatory challenges. This hyperactivity of the immune system translates into increased levels of central and circulating cytokines that would induce the abovementioned negative behavioural and psychological features of the sickness behaviour. Therefore, inflammation can be posited as the pathway between stress and disease, underlying the vulnerability to stress-related disorders.

### 3.4. Role of inflammation in stress induced vulnerability to drug addiction

As previously discussed (see Section 2.3), stress experiences have a solid impact on increasing the vulnerability to develop drug use disorders and addiction. Recent scientific literature points to immune mechanisms as possibly underlying this phenomenon.

Stress stimulates the immune system and promotes the release of inflammatory markers in the periphery and inside the immune system (Frank et al., 2016; 2019; Powell et al., 2013). This, in turn, can enhance the rewarding response to drugs through its action on the HPA axis. Cytokines induce a direct activation of the neurohormonal stress response of the HPA axis (Rivest, 2001; Silverman et al., 2004) and this activation leads to the release of CRF that modulates DA functioning through its action on the VTA (Boyson et al., 2014). Chronic elevation of CRF levels caused by enhanced inflammatory signal would alter normal function of dopaminergic neurons within the reward pathway, causing long-term neuroadaptations (Haass-Koffler & Bartlett, 2012). These changes would lead to increased hedonic response to different substances of abuse, including cocaine (Han et al., 2017).

Furthermore, inflammatory states can also increase the reinforcing properties of drugs. During drug cessation, there is an increase in CRF signalling within the extended

amygdala that has been associated with the emotional negative state that appears during drug withdrawal (Tunstall & Carmack, 2016). Increases in CRF induced by pro-inflammatory markers can activate and sensitize CRF receptors within the extended amygdala (Roberto et al., 2017), progressively enhancing states of anxiety, irritability and depressed mood during withdrawal. These negative states would drive the seek for the drug in order to "self-medicate" and mitigate these emotional disturbances (Koob & Volkow, 2010; Shalev et al., 2010), contributing to the loss of control over drug consumption and the chronification of the disorder (Zorrilla et al., 2014).

Finally, as introduced in Section 3.2, this repeated consumption of drugs, such as ethanol or cocaine, induces by itself an activation of the immune system, shifting it to a pro-inflammatory state. Moreover, this drug-induced inflammation could be amplified, as repeated contact with the drug may sensitize even further the immune system to subsequent insults (Alfonso-Loeches et al., 2010), including stress challenges (Niraula et al., 2017; Weber et al., 2019). Thus, chronic drug consumption would promote a general pro-inflammatory state with the corresponding "sickness behaviour", which again may also be promoting the recurrent consumption in order to relieve the discomfort. Lastly, the sustained inflammation induced by drug consumption could have neurotoxic effects that impair cognitive functions.

As a conclusion, in drug addiction, there is a vicious circle between stress, inflammation and drug consumption. The discovery of this central role of the immune system in substance-related disorders opens new therapeutic opportunities based on anti-inflammatory interventions focused on counteracting the negative consequences of stress and chronic drug consumption.

### 3.5. Anti-inflammatory interventions as a therapeutic approach in stress-related disorders

The recent knowledge on how inflammatory processes affect the vulnerability to several mental disorders, especially stress-related ones, have opened a window in the exploration of anti-inflammatory agents as preventive and therapeutic strategies (Kappelmann et al., 2018). For instance, treatment with non-steroidal anti-inflammatory drugs (NSAIDs) as a monotherapy or as adjuvant to antidepressants proved to be more effective than placebo in treating depression in a recent meta-analysis of randomized

controlled trials (Köhler et al., 2014, 2019). In the same line, the use of cytokine blockers, such as infliximab (antibody for TNF-α), improves the mood of patients being treated for inflammatory conditions such as psoriasis or inflammatory bowel disease (Shariq et al., 2018). In rodent models, treatment with an IL-6 antibody has been reported to prevent depression-like behaviour induced by an inflammatory stimulus or stress (Hodes et al., 2014), while treatment with indomethacin, an NSAIDs, reversed cognitive impairments induced by chronic SD stress (Duque et al., 2017).

Additionally, some non-pharmacological treatments that have demonstrated their therapeutic efficacy in the management of stress-related disorders also have an anti-inflammatory potential. There is growing evidence supporting that part of the clinical improvement produced by classical psychotherapies, such as cognitive behaviour therapy, and other new therapeutic approaches, such as mindfulness, can be explained given its potential to minimize inflammatory responses to stressful experiences (Lopresti, 2017; Rosenkranz et al., 2013). In this line, social support, which is considered an extra-therapeutic context in depression treatment (Park et al., 2014), has been proven to enhance resilience to stress and prevent the development of psychopathology, including depression (Ioannou et al., 2018; Ozbay et al., 2007). Once again, the protective effect of social support is associated to anti-inflammatory effects (Yang et al., 2014; Ysseldyk et al., 2018). This can be sustained by the release of the anti-inflammatory neuropeptide oxytocin (OT) (Li et al., 2017; McQuaid et al., 2014), which is released during social and physical contact and potentiates social behaviours (Carter, 2003).

There is also a growing interest in studying how dietary patterns can modulate inflammatory markers to determine if they could be an effective treatment for depression (Tolkien et al., 2018). However, two recent meta-analysis (Tolkien et al., 2018; Wang et al., 2019) and a large prospective study (Adjibade et al., 2017) failed to demonstrate the clinical benefit of an anti-inflammatory diet. On the other hand, a recent systematic review concluded that the administration of probiotics in clinical depressed patients was a promising adjuvant treatment capable of improving depressive symptomatology by increasing serotonin levels and decreasing levels of inflammatory markers (Wallace et al., 2017). In animal models, probiotics have been shown to

decrease the circulating levels of the peripheral cytokines such as IL-1, IL-6 and TNF-α, as well as central microglial activation (Ait-Belgnaoui et al., 2012, 2014).

Furthermore, exercise has shown to have positive effects in rodents and therapeutic effects in humans (Lucassen et al., 2010). Part of its beneficial action is caused by its ability to decrease inflammatory markers and oxidative stress by reducing the expression of TLR on monocytes and macrophages, with a subsequent decrease in the reactivity of these immune cells (Eyre & Baune, 2012; Gleeson et al., 2011). In addition to this anti-inflammatory potential, it also modulates monoamine metabolism, decreases basal levels of GCs and potentiates neuroplasticity, effects that are crucial for understanding the positive effect of physical exercise in stress-related disorders (Eyre & Baune, 2012).

# 2. AIMS AND HYPOTHESES

## 2. Aims and Hypotheses

As we have seen in the previous sections, stress is a central factor in the genesis of substance use disorders and addiction. Clinical studies constantly report that stressful experiences are a risk factor in all stages of the addictive cycle. This finding has been supported by basic research, which has also increased the knowledge on the neurobiological mechanisms underlying the stress-enhancement of drug effects. While many studies have linked changes in the reward pathway to stress-induced vulnerability, to date, few studies have evaluated the impact of immune alterations in the increased vulnerability to substance use disorders induced by stress. Knowing that stress-induced inflammation is crucial in the genesis of mood disorders, we considered that further research was necessary to unravel a possible role of the immune system in the increased risk to cocaine use disorders after stress exposure.

The main objective of the present Doctoral Thesis was to validate the inflammation theory of stress-induced vulnerability in drug addiction. To assess this objective, we first confirmed and characterized the pro-inflammatory potential of SD stress. Afterwards, we determined whether this pro-inflammatory response was mediating the enhancement in cocaine response using a CPP paradigm. Lastly, we evaluated whether interventions that have classically been reported to be effective buffers of stress consequences, such as physical activity or social and environmental enrichment, exerted this protective effect by anti-inflammatory actions.

The confirmation of the contribution of the immune system in the vulnerability to substance use disorders will open a novel research field, where inflammatory factors can be explored as bio-markers for vulnerability, and anti-inflammatory strategies as preventive and therapeutic interventions.

**Study 1.** The aim of the first study was to determine the role of the HPA axis in the long-lasting behavioral consequences of SD stress, such as anxiety-like behavior, increased CPP, and locomotor sensitization induced by cocaine. To this end, two antagonists of the CRF receptors were administered prior to the SD episodes, which blocked the neuroendocrine response of the HPA axis.

Hypotheses

- Animals that experienced repeated SD protocol will show long-lasting sensitization to the rewarding properties of cocaine and develop CPP with a subthreshold dose (1mg/kg) of the drug.
- Animals exposed to repeated SD protocol will display long-term anxiety-like behavior measured in the elevated plus maze and open field paradigms.
- Animals administered with high doses of cocaine (25mg/kg) will develop locomotor sensitization and display greater locomotor activity to a challenge dose of 10mg/kg than naïve animals with no previous contact with cocaine.
- Animals exposed to repeated SD protocol will develop long-term locomotor cross-sensitization and display higher locomotor activity in response to a challenge dose of cocaine than animals without stress experience.
- Pretreatment with CRF1 or CRF2 receptor antagonists before SD episodes will block the enhancement in cocaine reward induced by SD stress.
- Pretreatment with CRF1 or CRF2 receptor antagonists before SD will prevent the development of anxiety-like behavior in defeated mice.
- Pretreatment with CRF1 or CRF2 receptor antagonists before SD will prevent the development of locomotor cross-sensitization to cocaine.

**Study 2.** The aim of the second study was to determine whether central and peripheral inflammatory processes were mediating the long-lasting effects of SD stress in cocaine reward and in anxiety-like behavior. In order to fulfill this objective, we evaluated the inflammatory potential of SD and the effect of a pretreatment with an anti-inflammatory drug prior to stress episodes.

Hypotheses

- Animals exposed to repeated SD stress will display long-lasting anxiety-like behavior and acquire CPP induced by a subthreshold dose of cocaine.
- Animals exposed to repeated SD stress will display increased central and peripheral levels of the pro-inflammatory cytokine IL-6 after SD episodes.
- Exposition to SD stress will sensitize the neuroimmune axis of defeated mice, which will have an amplified inflammatory response after cocaine CPP.
- Animals pretreated with the anti-inflammatory indomethacin before each episode of SD will not develop anxiety-like behavior or CPP induced by a subthreshold dose of cocaine.
- The administration of the anti-inflammatory indomethacin before each episode of SD will prevent the increase of central and peripheral IL-6 levels in defeated mice.

**Study 3.** The aim of the third study was to determine if a pretreatment with the neuropeptide OT prior to SD episodes counteracts the long-lasting effects of social stress in cocaine reward, locomotor sensitization and anxiety-like behavior.

Hypotheses

- Animals pretreated with OT before SD will not develop long-lasting anxiety-like behavior and will not develop CPP induced by a subthreshold dose of cocaine (1mg/kg).
- Pretreatment with OT before SD will prevent the development of locomotor cross-sensitization to cocaine.
- Animals in both stress conditions (SD and non-stressed) will develop CPP induced by an effective dose of cocaine (10mg/kg).
- Animals pretreated with OT in both stress conditions (SD and non-stressed) will require fewer sessions to extinguish the conditioned preference induced by 10mg/kg cocaine.
- Animals exposed to SD protocol will acquire cocaine (0.5mg/kg/infusion) self-administration behavior in greater proportion and self-administrate more cocaine than non-stressed animals.
- Animals pretreated with OT prior to SD episodes will not present enhanced acquisition of cocaine self-administration behavior.
- Animals pretreated with OT in both conditions (SD and non-stressed) will require fewer extinction sessions to extinguish cocaine-seeking behavior in the self-administration paradigm.

**Study 4.** This study aimed to determine if positive social housing, such as being housed in pairs with a female or with a familiar conspecific, is a protective intervention to counteract the long-lasting increase in cocaine reward, pro-inflammatory markers and anxiety-like behavior induced by SD stress. Additionally, we aimed to determine if the physiological mechanism underlying the protective effect of these social interventions is dependent on OT.

Hypotheses

- Socially defeated animals housed in positive social housing conditions will not develop long-lasting anxiety-like behavior and will not develop CPP induced by a subthreshold dose (1mg/kg) of cocaine.
- Socially defeated animals housed in positive social housing conditions will not display increased central levels of IL-6 after CPP protocol.
- Animals housed in positive social housing conditions will have increased plasmatic OT levels when compared to animals living in standard housing conditions.
- The chronic administration of the OT antagonist atosiban will block the protective effect of positive social housing.

**Study 5.** This study aimed to determine if environmental enrichment is an effective intervention to counteract the long-lasting increase in cocaine reward induced by SD stress. Additionally, we aimed to determine if this protective effect is related to an anti-inflammatory effect of the intervention.

Hypotheses

- Animals exposed to a repeated SD protocol and housed in an enriched environment will not develop anxiety-like behavior or CPP induced by a subthreshold dose (1mg/kg) of cocaine.
- Environmental enrichment will exert an anti-inflammatory effect and animals housed under this condition will display lower plasmatic IL-6 levels when compared to animals under standard housing conditions.

**Study 6.** The objective of the present study was to determine if physical activity, specifically voluntary wheel running, is an effective intervention in counteracting the long-lasting increase in cocaine reward induced by SD stress.

Hypotheses

- Sedentary animals exposed to repeated SD stress will acquire CPP induced by a subthreshold dose of cocaine.
- Sedentary animals exposed to repeated SD stress will display increased central and peripheral levels of the pro-inflammatory cytokine IL-6.
- SD animals in a regimen of increased physical activity, with permanent or intermittent access to running wheels, will not develop CPP induced by a subthreshold dose of cocaine.
- SD animals in a regimen of increased physical activity, with permanent or intermittent access to running wheels, will be protected against inflammatory potential of SD stress and will not display increased IL-6 levels.
- Voluntary wheel running is an alternative reinforcer that can induce place preference and counteract the development of CPP induced by an effective dose (3mg/kg) of cocaine.

**Study 7. (Icahn School of Medicine at Mount Sinai).** The aim of this study was to determine if social stress experiences can compromise the permeability of the BBB and let peripheral immune cytokines and cells enter into the brain. Additionally, we aimed to determine if the level of loosening of the BBB can predict stress susceptibility or resilience.

Hypotheses

- Animals exposed to the chronic SD protocol will display reduced levels of claudin-5 (cldn5) mRNA in the nucleus accumbens when compared to the levels of non-stressed animals.
- Animals exposed to chronic SD protocol will have infiltrated peripheral immune cells in their brain parenchyma and this phenomenon will not be observed in non-stressed animals.
- Animals exposed to SD stress will display social avoidance as a psychological consequence induced by stress experience.
- Among SD animals, the greater affectation of the integrity of the BBB (lower levels of cldn5 and greater peripheral cell infiltration) will correlate with higher susceptibility to the psychological consequences of stress in the form of increased levels of social avoidance.

# 3. RESULTS

# Study 1

Antagonism of corticotropin-releasing factor CRF1 receptors blocks the enhanced response to cocaine after social stress.

**Ferrer-Pérez, C.,** Reguilón, M. D., Manzanedo, C., Aguilar, M. A., Miñarro, J., & Rodríguez-Arias, M.

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Behavioural pharmacology

# Antagonism of corticotropin-releasing factor $CRF_1$ receptors blocks the enhanced response to cocaine after social stress

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ABSTRACT

Numerous studies have shown that social defeat stress induces an increase in the rewarding effects of cocaine. In this study we have investigated the role played by the main hypothalamic stress hormone, corticotropin-releasing factor (CRF), in the effects that repeated social defeat (RSD) induces in the conditioned rewarding effects and locomotor sensitization induced by cocaine. A total of 220 OF1 mice were divided into experimental groups according to the treatment received before each social defeat: saline, 5 or 10 mg/kg of the nonpeptidic corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526, or 15 or 30 μg/kg of the peptidic corticotropin-releasing factor $CRF_2$ receptor antagonist Astressin$_2$-B. Three weeks after the last defeat, conditioned place preference (CPP) induced by 1 mg/kg of cocaine was evaluated. Motor response to 10 mg/kg of cocaine was also studied after a sensitization induction. Blockade of corticotropin-releasing factor $CRF_1$ receptor reversed the increase in cocaine CPP induced by social defeat. Conversely, peripheral corticotropin-releasing factor $CRF_2$ receptor blockade produced similar effects to those observed in socially stressed animals. The effect of RSD on cocaine sensitization was again blocked by the corticotropin-releasing factor $CRF_1$ receptor antagonist, while peripheral $CRF_2$ receptor antagonist did not show effect. Acute administration of Astressin$_2$-B induced an anxiogenic response. Our results confirm that CRF modulates the effects of social stress on reinforcement and sensitization induced by cocaine in contrasting ways. These findings highlight CRF receptors as potential therapeutic targets to be explored by research about stress-related addiction problems.

## 1. Introduction

Drug addiction can be considered a multifactorial disorder of chronic relapse as a result of the interaction of biological and environmental factors, characterized by a loss of control over use of the drug (Kalivas and Volkow, 2005; Koob, 2010; Koob and Volkow, 2009; Volkow et al., 2015). It has been repeatedly demonstrated that adverse life experiences can render individuals more prone to addictive substances of abuse and make them more vulnerable to relapse after periods of detoxification (Le Moal, 2009; Miczek et al., 2008; Sinha et al., 2011). Thus, stress is considered a risk factor that can influence all stages of drug addiction, as it plays a role in initiation, maintenance, escalation of intake and relapse (Burke and Miczek, 2015; Kalivas and Volkow, 2005; Koob, 2010; Sinha et al., 2011; Logrip et al., 2011, 2012).

Social interaction is known to be the main source of stress in human beings (Dickerson and Kemeny, 2004; Tamashiro et al., 2005). An animal model of social stress should closely mimic real-life situations in human life (Neisewander et al., 2012); in this context, social defeat in an agonistic encounter between rodents is a model of ecological validity for recreating experiences such as bullying, physical abuse or subordination stress (Björkqvist, 2001; Lu et al., 2003; Miczek et al., 2008; Tornatzky and Miczek, 1993). After being exposed to repeated social defeat, rodents show a range of depression-like symptoms such as anhedonia, social withdrawal and metabolic syndrome (weight gain and insulin and leptin resistance) (Meerlo et al., 1996; Von Frijtag et al., 2000). It has repeatedly been shown that exposure to different procedures of social defeat increases the rewarding and reinstating effects of psychostimulant drugs, such as cocaine, in the self-administration (SA) and conditioned place preference (CPP) paradigms (Aguilar et al., 2013; Miczek et al., 2008; Neisewander et al., 2012). Defeated animals also show an enhanced sensitivity to cocaine-induced hyperactivity (Nikulina et al., 1998; Nikulina et al., 2004) and dopaminergic cross-sensitization, with an augmented behavioral response to subsequent doses of cocaine after repeated drug exposure (Kalivas et al., 1998; Piazza and Le Moal, 1998; Steketee and Kalivas, 2011). These effects

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are associated with increased dopamine transmission in the corticolimbic system (Steketee and Kalivas, 2011), including projections from the ventral tegmental area to the medial prefrontal cortex and the nucleus accumbens (Pierce and Kalivas, 1997; Steketee and Kalivas, 2011), which are the main structures implicated in the rewarding and motor effects of psychostimulants (Koob, 2009; Sinha, 2008).

These changes in the corticolimbic system may be mediated by the action of neuropeptide corticotropin-releasing factor (CRF), which interacts with two G-protein-coupled corticotropin-releasing factor CRF receptors, type 1 (corticotropin-releasing factor $CRF_1$ receptor) and type 2 (corticotropin-releasing factor $CRF_2$ receptor), both positively coupled to adenylate cyclase through stimulatory G-proteins (Binder and Nemeroff, 2010; Hauger et al., 2009; Zorrilla and Koob, 2004, 2010) with actions at central and peripheral levels (Hauger et al., 2006; Heinrichs and Koob, 2004). This factor is known as the principal mediator of a wide range of both acute and chronic neuroendocrine and behavioral responses to stress (Logrip et al., 2011). Corticotropin-releasing factor activates the hypothalamic-pituitary-adrenal axis and stimulates the release of glucocorticoids (Bale and Vale, 2004). In addition, corticotropin-releasing factor axons project to extrahypothalamic areas such as the extended amygdala and ventral tegmental area, thereby modulating dopamine function and causing neuroadaptations in dopamine neurons in the corticolimbic pathway (Haass-Koffler and Bartlett, 2012; Saal et al., 2003; Wanat et al., 2008). This especially relevant during withdrawal (Zorrilla et al., 2014), when corticotropin-releasing factor plays a key role in anxiety-like effects and helps to explain the negative reinforcement processes that drive the compulsivity of addiction (Koob, 2008).

The aim of this investigation was to study the role of both corticotropin-releasing factor receptors in the effects of social stress on the conditioned rewarding effects and locomotor sensitization induced by cocaine. To achieve our objective, we administrated a pretreatment of a peripheral injection of the nonpeptidic corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 or the peptidic corticotropin-releasing factor $CRF_2$ receptor antagonist $Astressin_2$-B before each social defeat. It should be noticed that corticotropin-releasing factor $CRF_1$ and 2 antagonists were injected peripherally. As CP-154,526 crosses the blood brain barrier we expected it to block central and peripheral corticotropin-releasing factor $CRF_1$ receptors. On the other hand, $Astressin_2$-B acts only on peripheral corticotropin-releasing factor $CRF_2$ receptors such as those located on the pituitary and other peripheral locations.

## 2. Material and methods

### 2.1. Animals

A total number of 220 male OF1 mice (Charles River, France) were delivered to our laboratory at 42 days of age. Experimental mice were housed in groups of four in plastic cages (27 × 27× 14 cm) during the entire experimental procedure. To reduce their stress levels in response to experimental manipulations, mice were handled for 5 min per day on each of the 3 days prior to initiation of social defeat experiences. Aggressive opponents were individually housed in plastic cages (21 × 32 × 20 cm) for a month prior to initiation of the experiments in order to heighten aggression (Rodrıguez-Arias et al., 1998). All mice were housed under the following conditions: constant temperature; a reversed light schedule (white light on 8:00–20:00 h); and food and water available ad libitum, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local Ethics Committee for Experimentation and Animal Welfare of the University of Valencia in January 2012 (approval ID A1302084466177).

### 2.2. Drugs

The corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 (Bio-Gen, Madrid, Spain) was injected intraperitoneally at a dose of 5 or 10 mg/kg 30 min before each social defeat. These doses have previously been shown by other researches to not affect locomotor activity (Przegaliński et al., 2005). A dose of 30 or 15 μg/kg of the corticotropin-releasing factor $CRF_2$ receptor antagonist $Astressin_2$-B (Bio-Gen, Madrid, Spain) was also injected intraperitoneally 30 min before each social defeat following the procedure developed by Rivier and co-workers (Rivier et al., 2003). For CPP and sensitization studies doses of 1, 10 or 25 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Madrid, Spain) were used. The lowest dose of cocaine was selected on the basis of previous CPP studies showing 1 mg/kg to be a threshold dose (Arenas et al., 2014; Montagud-Romero et al., 2014; Vidal-Infer et al., 2012). All the treatments were adjusted in a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs. In the case of CP-154,526, in order to achieve a complete suspension of the product, the solvent Tween-80 was added with a maximum concentration of 10%. We have previously tested the effect of Tween-80 in a control group and did not observe any effect on the development of CPP (Manzanedo et al., 2004).

### 2.3. Experimental design

The experimental design is depicted in Table 1. In the first study the role of corticotropin-releasing factor $CRF_1$ and corticotropin-releasing factor $CRF_2$ receptors in the effects that social defeat induce on the conditioned rewarding effects of cocaine (1 mg/kg) was evaluated. Mice were divided into ten experimental groups according to the treatment received before each social defeat (RSD) or exploration (EXP): physiological saline, the corticotropin-releasing factor $CRF_1$ receptor antagonist CP- 154,526 (5 or 10 mg/kg) or the corticotropin-releasing factor $CRF_2$ receptor antagonist $Astressin_2$-B (15 or 30 μg/kg). The CPP procedure was initiated three weeks after the last social defeat. Ten days after extinction of the CPP, the mice in each group were assigned to a cocaine (25 mg/kg) or saline condition and were injected for three consecutive days with a daily dose of the corresponding treatment. The sensitization study was performed in an additional set of mice 3 weeks after the exploration or RSD experiences. A sample of 68 mice was divided into four experimental groups according to the treatment received before each social defeat (RSD) or exploration (EXP): physiological saline, the corticotropin-releasing factor $CRF_1$ receptor antagonist CP- 154,526 (10 mg/kg) or the corticotropin-releasing factor $CRF_2$ receptor antagonist $Astressin_2$-B (30 μg/kg).

The last study was designed to evaluate the behavioral effect of an acute dose of 30 μg/kg $Astressin_2$-B on the open field and the elevated plus maze (EPM). The mice previously treated with the corticotropin-releasing factor $CRF_2$ receptor antagonist in the CPP experiment were used in this procedure, which took place after the end of the CPP test.

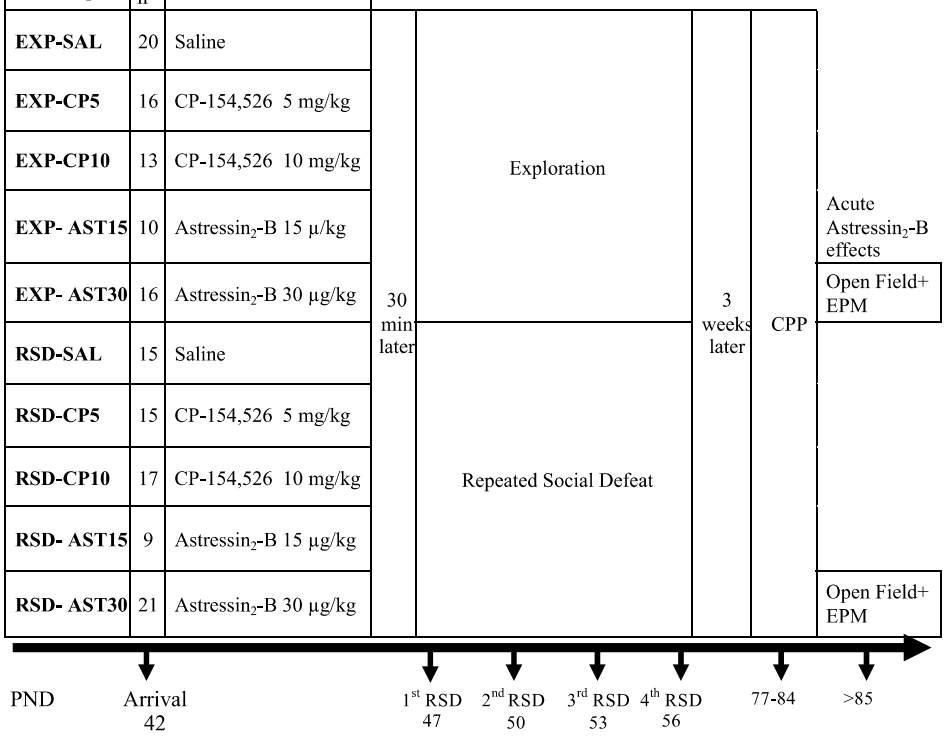
### 2.4. Apparatus and procedures

#### 2.4.1. Procedure of social defeat- RSD

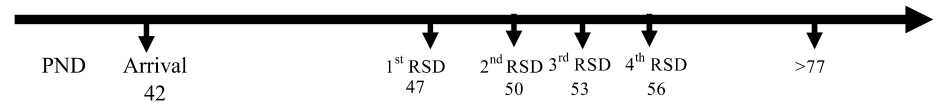
Repeated Social Defeat (RSD) procedure consisted of four episodes of social defeat, on post-natal days 47, 50, 53 and 56. Each episode consisted of three phases, which began by introducing the “intruder” (the experimental animal) into the home cage of the “resident” (the aggressive opponent) for 10 min (Tornatzky and Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus allowing instigation and provocation (Covington and Miczek, 2001). The wire mesh was then removed from the cage to allow confrontation between the two animals for a 5-min period. In the third phase, the wire mesh was

**Table 1**
Experimental design.

| Groups | n= | Treatment |
|---|---|---|
| **EXP-SAL** | 20 | Saline |
| **EXP-CP5** | 16 | CP-154,526 5 mg/kg |
| **EXP-CP10** | 13 | CP-154,526 10 mg/kg |
| **EXP- AST15** | 10 | Astressin$_2$-B 15 µ/kg |
| **EXP- AST30** | 16 | Astressin$_2$-B 30 µg/kg |
| **RSD-SAL** | 15 | Saline |
| **RSD-CP5** | 15 | CP-154,526 5 mg/kg |
| **RSD-CP10** | 17 | CP-154,526 10 mg/kg |
| **RSD- AST15** | 9 | Astressin$_2$-B 15 µg/kg |
| **RSD- AST30** | 21 | Astressin$_2$-B 30 µg/kg |




| | | | | | |
|---|---|---|---|---|---|
| **EXP-SAL** | 17 | Saline | 30 min later | Exploration | 3 weeks later; Locomotor Sensitization |
| **RSD-SAL** | 20 | Saline | | Repeated Social Defeat | |
| **RSD-CP10** | 16 | CP-154,526 10 mg/kg | | | |
| **RSD-AST30** | 15 | Astressin$_2$-B 30 µ/kg | | | |




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; Rodrıguez-Arias et al., 1998). All agonistic encounters were videotaped to confirm social defeat in our experimental mice.

### 2.4.2. Conditioned Place Preference-CPP

For place conditioning, twelve identical Plexiglas boxes with two equal sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a gray central area (13.8 cm length × 31.5 cm width × 34.5 cm height) were used. The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the position of the animal and its crossings from one compartment to the other to be recorded. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

Place conditioning consisted of three phases and took place during the dark cycle (Maldonado et al., 2006) following an unbiased procedure in initial spontaneous preference terms (for a detailed explanation of the procedure, see Daza-Losada et al. (2009)). Mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day over 3 days (on post-natal days 77–84). On day 3, the time spent by the animal in each compartment during a period of 900 s was recorded. A total of 19 mice that showed a strong unconditioned aversion (less than 33% of the time spent in both compartments) or preference (more than 67%) for any compartment were excluded from the study. In each group, half the mice received the drug or vehicle in one compartment and the other half in the other compartment. After assigning compartments, an analysis of variance revealed no significant differences between the time spent in the drug-paired and vehicle-paired compartments during the Pre-C phase. In the CPP induced by cocaine, mice underwent two pairings per day: subjects received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, received cocaine immediately before being confined to the drug-paired compartment for 30 min.

The central area was never used during conditioning and was blocked by guillotine doors. During the third phase, or post-conditioning (Post-C), the guillotine doors separating the two compartments were removed and the time spent by the untreated mice in each compartment during a 900 s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced by the drug. A CPP score value was calculated for each mouse as the difference between time spent in the drug-paired compartment during the Post-C minus the Pre-C test. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the induction of an aversion.

All groups in which a preference for the drug-paired compartment was established underwent an extinction session every 72 h, which consisted of placing mice in the apparatus (without the guillotine doors separating the compartments) for 15 min. This was repeated until the time spent in the drug-paired compartment by each group was similar to that of Pre-C. Thus, all the animals in each group underwent the same number of extinction sessions, independently of their individual scores. Extinction of CPP was always confirmed 24 h after the last extinction session. In addition, the time required for preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test. Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was considered to be extinguished when a mouse spent 370 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 370 s). When the preference was not extinguished in an animal, it was assigned the number of days required for extinction for the group as a whole.

The effects of non-contingent administration of a priming dose of cocaine were evaluated 24 h after confirmation of extinction. Reinstatement tests were the same as for Post-C (free ambulation for 15 min), except that mice were tested 15 min after administration of the drug (half of the dose used for conditioning). After this first reinstatement test, the groups that demonstrated reinstatement—i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and last extinction tests (confirmed with a Student's *t*-test)—were retested until a new extinction was confirmed. The following day, the effects of the priming (a quarter of the dose used for conditioning) on reinstatement of place preference were evaluated following the procedure described previously. This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

#### 2.4.3. Locomotor sensitization testing

Locomotor sensitization induced by cocaine was measured evaluating the movement of mice inside the cage by means of photocell cuts. Ten days after extinction of the CPP, mice were assigned to a cocaine or saline condition and were injected with a dose of 25 mg/kg per day of cocaine or saline on three consecutive days in their home cages. After five days with no treatment all mice were injected with 10 mg/kg cocaine and placed for the first time in the actimeter. Locomotor activity was automatically measured by an actimeter (CIBERTEC S.A., Spain) consisting of eight cages (33 × 15 × 13 cm), each with eight infrared lights located in a frame around the cage. In this apparatus, beams are positioned on the horizontal axis 2 cm apart, at a height just above the bottom of the cage (body level of mice). The different frames are placed 4 cm apart and, since they are opaque, prevent animals from seeing each other while allowing them to hear and smell conspecifics being tested at the same time.

#### 2.4.4. Elevated plus maze-EPM

The elevated plus maze (EPM) test was carried out essentially following the procedure described by Daza-Losada and co-workers (Daza-Losada et al., 2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and 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. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The measurements recorded during the test period were number of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. The percentage of time spent in the open arms and the number of open arm entries are generally used to characterize the anxiolytic effects of drugs. In addition, the number of closed and total entries indicates motor activity.

#### 2.4.5. Open-field test

Mice were habituated to an open field (30.5 × 29 × 35 cm) dark cage and were allowed to explore freely for an hour. Immediately afterwards, mice were injected with 30 $\mu$g/kg of the corticotropin-releasing factor $CRF_2$ receptor antagonist Astressin$_2$-B and immediately placed again in the open-field area for 30 min. Activity was tracked and analyzed using EthoVision XT software (Noldus Information Technology, http://www.noldus.com) to determine the total distance covered and the percentage of time spent in the center of the cage.

### 2.5. Statistical analyses

For the CPP data, the CPP score was analyzed with a mixed three-way ANOVA, with two between-subjects variables—Pre-treatment, with five levels (Saline, CP5, CP10, Astressin$_2$-B 15 and Astressin$_2$-B 30), and Stress, with two levels (RSD and EXP). In all cases, post-hoc comparisons were performed with Bonferroni tests. In addition, the groups showing CPP, extinction and reinstatement values were analyzed by a Student's *t*-test. Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was considered to be extinguished when a mouse spent 380 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 370 s). When the preference was not extinguished in an animal, it was assigned the number of days required for extinction for the group as a whole. The time required for the preference to be extinguished in each animal was analyzed by means of the Kaplanen achieved, preference was considered to there were differences among groups (Daza-Losada et al., 2009).

The data for locomotor sensitization test were analyzed using a two-way ANOVA (for the groups that did not receive CRF antagonist treatment) with two between-subjects variables - Stress (RSD and EXP) and Drug (Saline and Cocaine) -and a two way ANOVA (for all the groups that experience RSD) with two between-subjects variables: Pre-treatment (saline, CP10 and Astressin$_2$-B30) and Drug (Saline and Cocaine). Data for each measure in the anxiety test (elevated plus maze and the open field) were analyzed with an ANOVA with two between-subjects variables: Stress (RSD and EXP) and Drug (Saline and Astressin$_2$-B). All post-hoc comparisons were performed using Bonferroni tests.

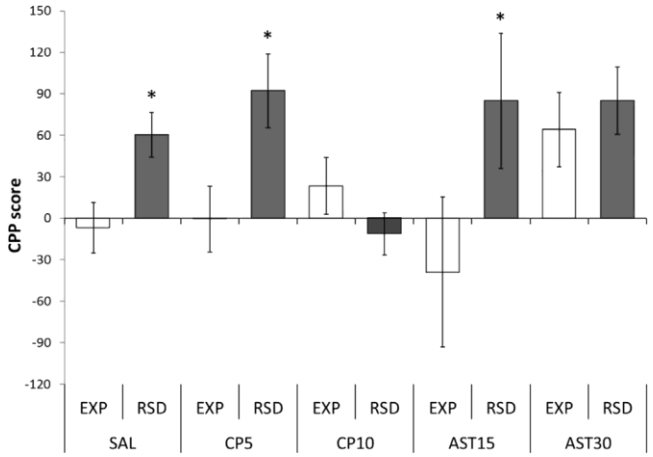



**Fig. 1.** $CRF_1$ receptor antagonist administration before each social defeat blocks the acquisition and reinstatement of the CPP induced by 1 mg/kg of cocaine in defeated mice. During the conditioning phase, defeated (RSD) or control animals (EXP) were divided into the following ten pre-treatment groups: saline (EXP-SAL n = 20; RSD-SAL n = 15); 5 (EXP-CP5 n = 16; RSD-CP5 n = 15) or 10 mg/kg (EXP-CP10 n = 13; RSD-CP10 n = 17) of the corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526; and 15 (EXP-AST15 n = 10; RSD-AST15 n = 19) or 30 μg/kg (EXP-AST30 n = 16; RSD-AST30 n = 21) of the corticotropin-releasing factor $CRF_2$ receptor antagonist Astressin$_2$-B. Data presented as mean values ± S.E.M. of the CPP score calculated as the time spent in the drug-paired compartment on the Pre-C minus the time spent on the Post-C test. *P < .05; **P < .01, ***P < .001 significant difference in the CPP score with respect to the corresponding EXP group.

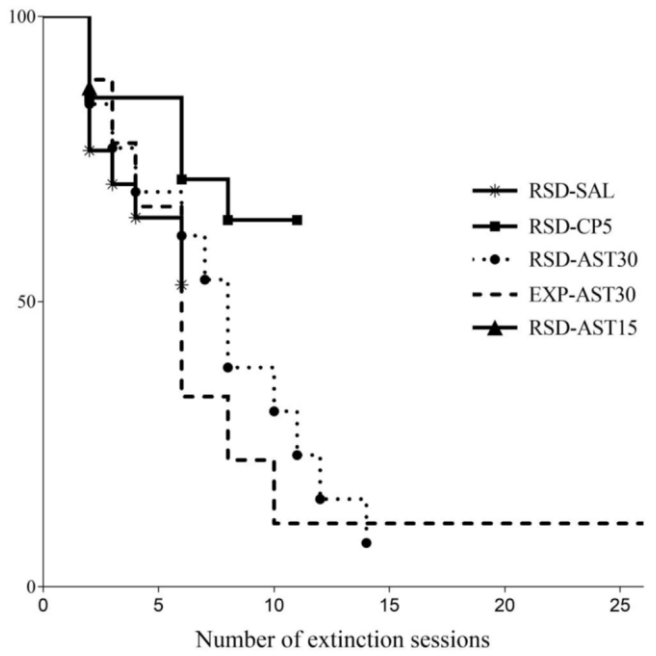



**Fig. 2.** The lowest dose of $CRF_1$ receptor antagonist and the highest dose of $CRF_2$ receptor antagonist before each social defeat increase the number of sessions needed to extinguish the CPP induced by 1 mg/kg of cocaine. Data presented as a survival curve showing the percentage animals in each group that meet the extinction criteria.

## 3. Results

### *3.1. Corticotropin-releasing factor $CRF_1$ receptor antagonist blocks the increase in the conditioned rewarding effects of cocaine (1 mg/kg) induced by social defeat stress*

ANOVA of the CPP data (see Fig. 1) showed a significant effect of the variable Stress [F (1123) = 11,104; P < .001] and the interaction Pre-Treatment x Stress [F (4123) = 3.048; P < .02]. Socially defeated animals pretreated with saline (RSD-SAL) achieve significant higher CPP scores (P < .05) compared to non-stressed group (EXP-SAL). That effect that was blocked in defeated mice treated with the highest dose of the corticotropin-releasing factor $CRF_1$ antagonist (RSD-CP10). On the other hand, defeated animals pretreated with the lowest dose of $CRF_1$ antagonist or the $CRF_2$ antagonist achieved significant higher CPP scores (P < .05) compared with their corresponding non-stressed control groups (EXP-CP5 and EXP-AST15). Finally, no differences were registered on CPP scores between exploration (EXP-AST30) and social defeated animals pretreated with the highest dose of the Astressin$_2$-B antagonist (RSD-AST30), as both developed CPP.

All groups that showed CPP were subjected to extinction sessions, and the Kaplan–Meier analysis revealed that more time was required to achieve extinction in the RSD-CP5 ($\chi 2$ = 10.721; P < .001) and RSD-AST30 ($\chi 2$ = 4.955; P < .05) groups than in the RSD-SAL group (see Fig. 2). On the other hand, RSD-AST15 group required less extinction sessions (only 2), than the RSD-SAL group ($\chi 2$ = 10.235 P < .001).

Reinstatement of the preference was obtained in the RSD-SAL (P < .05) and RSD-AST30 (P < .01) groups after a priming dose of 0.5 mg/kg of cocaine. No differences were observed in the time needed to extinguish this preference, and neither of these two groups showed reinstatement after priming with 0.25 mg/kg cocaine.

### *3.2. A corticotropin-releasing factor $CRF_1$ receptor antagonist reduces the increased cocaine locomotor sensitization induced by social defeat stress*

The results of the study of cocaine-induced sensitization of motor activity during the first period of 10 min are presented in Fig. 3. The

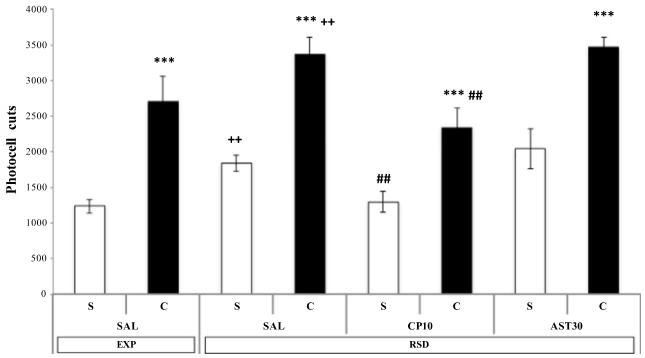



**Fig. 3.** $CRF_1$ receptor antagonist administration before each social defeat blocks the increased locomotor sensitization to cocaine induced by social defeat stress. During the induction phase, half of the animals in each group received a pretreatment with saline (S) (S-EXP-SAL n = 8; S-RSD-SAL n = 10; S-RSD-CP10 n = 8; S = RSD-AST30 n = 8) and the other half received 25 mg/kg cocaine per day (C) (C-EXP-SAL n = 9; C-RSD-SAL n = 10; C-RSD-CP10 n = 8; C-RSD-AST30 n = 7) on three consecutive days. Data presented as mean values ± S.E.M. during the first 10 min period of locomotor activity in photocell cuts. *** P < .001 significant difference with the corresponding saline group; ++ P < .001 significant difference with respect to the corresponding Exploration group; ## P < .001 significant difference with respect to the corresponding RSD group treated with saline.

ANOVA for the groups treated with saline revealed an effect of the variables Stress [F (1,33) = 7.309; P < .01] and Cocaine [F (1,33) = 40.505; P < .001]. Confirming the development of motor sensitization, we observed that all the animals treated with cocaine (C-EXP-SAL and C-RSD-SAL) were more active than those treated with saline (P < .001). Interestingly, RSD induced cross-sensitization and increased sensitization to cocaine, since the defeated groups (S-RSD-SAL and C-RSD-SAL) exhibited higher levels of motor activity than their non-stressed counterparts (P < .01).

ANOVA for the defeated groups showed an effect of the variables Pre-treatment [F (2,45) = 7.851; P < .01] and Cocaine [F (1,45) = 45.675; P < .001], and again confirmed the development of motor

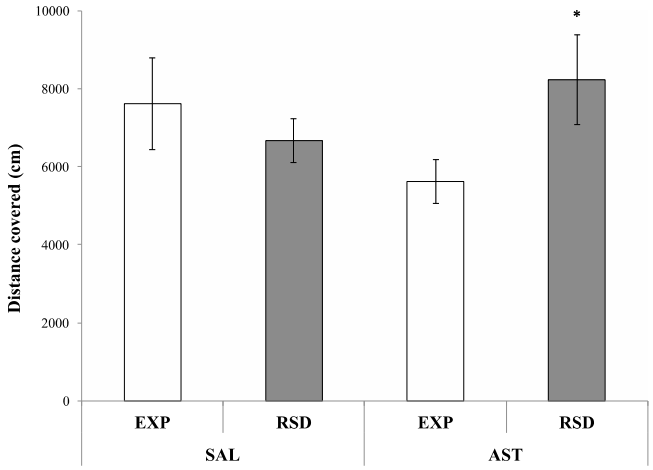



**Fig. 4.** Acute administration of 30 μg/kg Astressin$_2$-B increases locomotor activity. The bars represent the total distance covered (cm) in a 30 min period during the open field test. Half of the animals in each group received an acute injection with saline (EXP-SAL n = 10; DSR-SAL n = 8) or a 30 μg/kg dose of Astressin$_2$-B (EXP-AST n = 10; DSR-AST n = 8). Data presented as mean values test ± S.E.M. * P < .05 significant difference versus the corresponding exploration group.

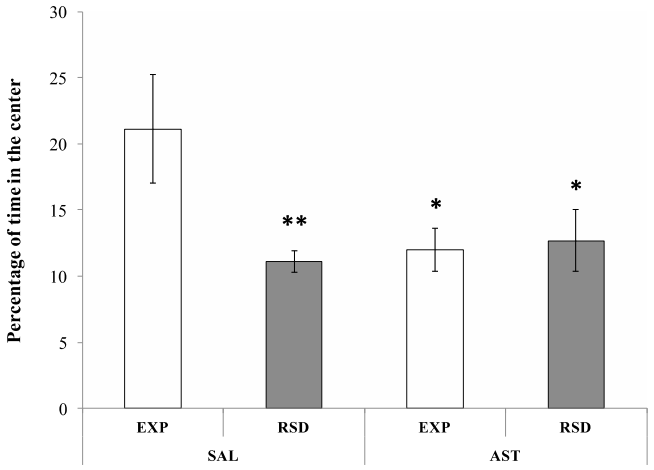



**Fig. 5.** Social defeat and acute administration of 30 μg/kg of Astressin$_2$-B decrease the percentage of time spent in the center of the open field. Half of the animals in each group received an acute injection with saline (EXP-SAL n = 10; DSR-SAL n = 8) or a 30 μg/kg dose of Astressin$_2$-B (EXP-AST n = 10; DSR-AST n = 8). Data presented as mean values ± S.E.M. *P < .05; **P < .01 significant difference in the percentage.

sensitization, since all the animals treated with cocaine (C-EXP-SAL and C-RSD-SAL) were more active than those treated with saline (P < .001). In addition, the groups treated with the corticotropin-releasing factor CRF$_1$ receptor antagonist showed significantly lower activity than those treated with saline or the corticotropin-releasing factor CRF$_2$ receptor antagonist (P < .05 and P < .01, respectively).

### 3.3. Acute Astressin$_2$-B increases anxiety behavior

#### 3.3.1. Open Field

ANOVA performed for the data of total distance covered during a 30 min period showed an effect of the interaction Stress x Astressin$_2$-B [F (1,34) = 3.656; P < .05]. Post-hoc comparisons showed that stressed animals that received an acute administration of Astressin$_2$-B covered more distance (P < .05) than those that were not stressed, a difference that was not observed in animals treated with saline. These results are represented in Fig. 4.

ANOVA performed for the data of the percentage of time spent in the center of the open field revealed an effect of the interaction Stress x Astressin$_2$-B [F (1,34) = 3.608; P < .05]. Post-hoc comparisons showed that socially defeated mice, whether treated or not with Astressin$_2$-B, spent a lower percentage of time in the center of the cage (P < .05) with respect to non-stressed saline-treated animals. In addition, Astressin$_2$-B decreased the percentage of time non-stressed animals spent in the center of the apparatus (P < .01). Results are represented in Fig. 5.

#### 3.3.2. Elevated plus maze

The data of the EPM test are presented in Table 2. ANOVA for the percentage of time spent in the open arms revealed a significant effect of the variables Stress [F (1,34) = 6.33: P < .05]; Pretreatment [F (1,34) = 6.680: P < .05]; and interaction of Stress x Pretreatment [F (1,34) = 3.91: P < .05]. Socially defeated animals pretreated with Astressin$_2$-B spent a lower percentage of time in the open arms than those stressed but pretreated with saline (P < .01 in all cases).

ANOVA for the number of open arm entries (Open Entries) revealed a significant effect of the variables Stress [F (1,34) = 7.35: P < .01] and Pre-treatment [F (1,34) = 10.042 P < .01]. Simple effects analyses showed that socially defeated animals performed less entries onto the open arms than non-stressed animals (P < .001). Mice pretreated with Astressin$_2$-B also showed less entries onto the open arms when compared with those receiving saline (P < .05). ANOVA also revealed a significant effect of the variable Stress on the percentage of entries onto the open arms [F (1,34) = 7.53: P < .01]. Socially defeated animals displayed a lower percentage of entries onto the open arms than those in the exploration group (P < .01).

**Table 2**
Acute administration of 30 μg/kg of Astressin$_2$-B induces anxiogenic responses in the elevated plus maze. Data are presented as mean values ± S.E.M. a P < .01; b P < .001 significant difference with respect to the corresponding Saline group, c P < .001, d P < .01 significant difference with respect to the corresponding Exploration group.

| | EXP | | RSD | |
|---|---|---|---|---|
| | SAL | Astressin$_2$-B | SAL | Astressin$_2$-B |
| % time OA | 35.3 ± 6.8 | 32 ± 5 | 32.4 ± 8 | 7.3 ± 8.1 [a] |
| Time in CA (s) | 158.3 ± 17 | 169.5 ± 12.4 | 166.6 ± 22.3 | 217.7 ± 29.3 |
| Open entries | 33.6 ± 3.4 | 27.2 ± 3.8 [b] | 24.3 ± 2.2 [c] | 12.1 ± 1.6 [b,c] |
| % entries OA | 68.3 ± 9.1 | 62 ± 6.6 | 44.9 ± 5[d] | 45.9 ± 8.1[d] |
| Closed entries | 39 ± 4.8 | 37.4 ± 5.2 | 41.1 ± 5.4 | 27.7 ± 3.4 |
| Total entries | 52.7 ± 4.2 | 48.6 ± 6.7 | 57.4 ± 6.6 | 31.9 ± 4.2 |

## 4. Discussion

The present study shows that peripheral administration of the nonpeptidic corticotropin-releasing factor CRF$_1$ receptor antagonist CP-154,526 before a social defeat inhibits the development of CPP induced with a subthreshold dose of cocaine and reverses the effects of social defeat on motor sensitization. Conversely, the administration of the peptidic corticotropin-releasing factor CRF$_2$ receptor antagonist Astressin$_2$-B exerted the opposite effects to those observed after corticotropin-releasing factor CRF$_1$ receptor blockade. The administration of the corticotropin-releasing factor CRF$_2$ receptor antagonist Astressin$_2$-B induced a stress response comparable to that observed after RSD. This stressing effect of Astressin$_2$-B was confirmed with the open field and EPM procedures. The present results suggest that the long-term effects of RSD on the conditioned rewarding effects of cocaine and the development of motor sensitization depend on central and peripheral CRF receptors. As previously reported, RSD increased the conditioned reinforcing effects of cocaine, as the CPP induced by a non-effective dose of this drug was acquired and reinstated in defeated mice. Equally, the present results confirm that motor sensitization can be induced by repeated pre-treatment with a high dose of cocaine, and that this phenomenon is modulated by stressful life experiences.

According to previous research, stress experiences have significant effects on the acquisition, expression, extinction and reinstatement of

CPP induced by cocaine (Logrip et al., 2011; Lu et al., 2003; Sanchez and Sorg, 2001). Previous studies in our laboratory have shown that mice exposed to social defeat exhibit a stronger CPP for the cocaine-paired compartment than non-stressed counterparts. Adult mice defeated during adolescence or adulthood developed a preference for the cocaine-paired compartment after being conditioned with a non-effective dose of the drug (1 mg/kg), and showed a reinstated preference after receiving only 0.5 mg/kg of cocaine (Montagud-Romero et al., 2016; Rodríguez-Arias et al., 2017). Similar results have been observed with alcohol and MDMA (García-Pardo et al., 2015; Rodriguez-Arias et al., 2016). Furthermore social defeat during an agonistic encounter in a neutral area prior to a reinstatement test has been shown to reinstate cocaine CPP in adult mice (Land et al., 2009; Titomanlio et al., 2013) and to increase susceptibility to cocaine-induced reinstatement of CPP (Do Couto et al., 2009). These results suggest that social stress induces sensitization of the reward system that makes mice more sensitive to these drugs.

In the present study, we have found that this augmented sensitivity to the conditioned rewarding effects induced by cocaine is modulated by corticotropin-releasing factor $CRF_1$ receptor. At the highest dose (10 mg/kg) the nonpeptidic corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 reversed the effects of social stress on the cocaine-induced CPP, since defeated mice did not develop CPP. While a lower dose (5 mg/kg) of CP-154,526 failed to block this effect (mice developed CPP), no reinstatement was achieved with a priming dose of 0.5 mg/kg, unlike in socially defeated animals pretreated with saline. It has to be taken into consideration that the corticotropin-releasing factor $CRF_1$ receptor antagonist has been dissolved with Tween-80, which has been probed to enhance drug delivery across the blood–brain barrier (Azhari et al., 2016). Although no studies have previously addressed this issue using the CPP and social stress paradigm, Boyson and co-workers (Boyson et al., 2011; Boyson et al., 2014) did observe that another corticotropin-releasing factor $CRF_1$ receptor antagonist (CP-376,395) -administrated peripherally or into the ventral tegmental area- reduced the escalation of cocaine self-administration after social defeat. Other researchers obtained similar results using corticotropin-releasing factor $CRF_1$ receptor antagonists and other kinds of stressors; for instance Shaham and co-workers (Shaham et al., 1998) found that administration of the corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 prior to a physical stressor (footshocks) significantly attenuated the reinstatement of self-administration of cocaine or heroin in trained rats, while Le and co-workers (Lê et al., 2000) also found an attenuation of footshock stress-induced reinstatement of ethanol self-administration. Using the CPP paradigm, Lu and co-workers (Lu et al., 2001) reported that CP-154,526 blocked the reinstatement of cocaine CPP induced by physical stress, but failed to block that induced by a priming dose of the drug.

After repeated, intermittent exposure to a psychostimulant an augmentation of drug response to motor activity is commonly observed, a phenomenon known as behavioral sensitization (Kalivas and Stewart, 1991; Steketee and Kalivas, 2011). On the other hand, several reports have pointed out that stress can induce a comparable enhancement of motor activity, which is termed cross-sensitization (Kalivas et al., 1998; Lu et al., 2001). In addition, social defeat induces an increase in the motor activity induced by amphetamine (Covington and Miczek, 2001; Miczek et al., 1999, 2011; Nikulina et al., 2004) or cocaine (Kikusui et al., 2005; Sasaki et al., 2015) and this phenomenon is related to neuroadaptations in the corticolimbic system of reward and its interaction with stress circuits, modulated by the CRF hormone (Haass-Koffler and Bartlett, 2012). Our results confirmed the development of a locomotor sensitization response to cocaine and cross-sensitization with RSD were observed. The group of defeated animals pretreated with saline showed a stronger motor response to cocaine than saline-treated controls. In addition, sensitization to cocaine was stronger in cocaine-treated defeated animals.

Although the highest dose of the corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 did not block cocaine-induced sensitization, blockade of corticotropin-releasing factor $CRF_1$ receptors significantly reduced cross-sensitization in socially defeated animals. Therefore, corticotropin-releasing factor $CRF_1$ receptor antagonist blocked the response induced by RSD, similarly to that observed with the CPP procedure. In a previous work, Giardino and co-workers (Giardino et al., 2012) showed that genetic deletion or pharmacological blockade of corticotropin-releasing factor $CRF_1$ receptor decreased the locomotor response to cocaine, although not to methamphetamine. In agreement with our results, Boyson and co-workers (Boyson et al., 2011) administered a corticotropin-releasing factor $CRF_1$ receptor antagonist into the ventral tegmental area before social stress and obtained a dose-dependent reduction of stress-induced locomotor sensitization to an acute cocaine challenge.

On the contrary, the blockade of peripheral corticotropin-releasing factor $CRF_2$ receptors before each social defeat produced the opposite effects on CPP and locomotor sensitization procedures depending on the dose employed. As $Astressin_2$-B is a peptidic antagonist it does not cross the blood brain barrier; it exerts its effects by specifically blocking pituitary CRF and peripheral receptors located in the heart, skeletal muscle, circulatory system and gastrointestinal tract (Hauger et al., 2006). Rivier and co-workers described that peripheral blockade of corticotropin-releasing factor $CRF_2$ receptors with $Astressin_2$-B in areas non protected by blood brain barrier modulate ACTH release after stress (Rivier et al., 2003).

We found that 30 $\mu$g/kg of $Astressin_2$-B induced by itself a comparable effect and potentiated the RSD stress on the CPP induced by cocaine. Both socially defeated and non-stressed mice treated with this corticotropin-releasing factor $CRF_2$ receptor antagonist developed preference to the cocaine-paired compartment. On the other hand, the lower dose of $Astressin_2$-B did not modify the effect of RSD on cocaine-induced CPP and did not induce any response in the non-stressed group. Related to locomotor sensitization, no effect was obtained in $Astressin_2$-B pretreated mice. These animals showed cross-sensitization and developed cocaine sensitization, similarly to defeated mice pretreated with saline.

There are few studies targeting cocaine and corticotropin-releasing factor $CRF_2$ receptor. Boyson and co-workers (Boyson et al., 2014) found that administration of $Astressin_2$-B directly into the ventral tegmental area 20 min before social defeat reversed the escalation and sensitization to dopamine release in the nucleus accumbens shell induced by cocaine. Equally, administration of another corticotropin-releasing factor $CRF_2$ receptor antagonist, anti-sauvagine-30, into the ventral tegmental area has been shown to block reinstatement of cocaine self-administration after physical stress (Wang et al., 2007). These results contrast with our findings, as we have observed that pretreatment with $Atressin_2$-B enhances the effects of social stress on drug behavior. The different routes of administration of the corticotropin-releasing factor $CRF_2$ receptor antagonists (central ventral tegmental area vs peripherally) and variations in the stress procedure used could explain these divergent results. In the same line of our results, several researchers have reported that knockout corticotropin-releasing factor corticotropin-releasing factor $CRF_2$ receptor mice have higher adrenocorticotropic-hormone and corticosterone levels in the blood due to hypersensitivity of the stress system (Bale et al., 2000; Bale and Vale, 2004; Logrip et al., 2011; Rivier et al., 2003), and that these mice display anxiety behavior in the EPM and open field (Wang et al., 2007). All of these findings point to a complex modulation of the stress response by $CRF_2$ receptor. One of the mechanisms through which antagonism of the $CRF_2$ extraneural receptors induced stressful responses in our study could be the inhibition of CRF release. Rivier and co-workers (Rivier and Rivier, 2014) observed that peripheral injection of $Astressin_2$-B slightly decreased CRF-induced ACTH release. Several authors have speculated that the corticotropin-releasing factor $CRF_2$ receptor acts as a pre-synaptic autoreceptor, inhibiting the release of CRF, which would, in turn, lead to decreased activation of the

corticotropin-releasing factor $CRF_1$ receptor (Valdez, 2009). Reul and Holsboer (Reul and Holsboer, 2002) hypothesized that this receptor plays a dual role of activation and inhibition of the stress response. On the one hand, the effect may be modulated by the moment at which the response takes place and by the location of the structure containing the receptor. Activation of corticotropin-releasing factor $CRF_2$ receptor induces an anxiogenic effect in the early phases of the response and an anxiolytic effect in the recuperation phase, thus antagonizing the effects of corticotropin-releasing factor $CRF_1$ receptor in structures like the paraventricular nucleus of hypothalamus, the bed nucleus of the stria-terminalis, the septal nuclei or the amygdala (Rivier et al., 2003).

Our results also showed that the highest dose of $Astressin_2$-B increased the rewarding effects of cocaine, since non-defeated animals treated with this dose developed CPP with a sub-threshold dose. We monitored the acute effects of 30 μg/kg of $Astressin_2$-B and found an increase of anxiety in the open field and EPM tests, an effect that was augmented by previous experience of social stress. It should be kept in mind that these tests took place after the end of the CPP procedure, which confirms that social stress induces long-lasting changes in the mouse brain. Although these effects could be interpreted as a stress response, previous reports have shown that $Astressin_2$-B does not promote anxiogenic-like behavior when administered centrally (Zorrilla et al., 2013). More experimental work is needed to clarify the effects of $Astressin_2$-B.

Finally, when analyzing the results obtained in our study, we must take into consideration that the non-peptidic corticotropin-releasing factor $CRF_1$ receptor antagonist CP-154,526 is capable of crossing the blood brain barrier and therefore can block corticotropin-releasing factor $CRF_1$ receptors in all locations, central and peripherally. On the other hand, the corticotropin-releasing factor $CRF_2$ antagonist $Astressin_2$-B is a peptidic compound that does not cross the BBB and therefore blocks only peripheral $CRF_2$ receptors. Importantly, CP-154,526 can block corticotropin-releasing factor $CRF_1$ receptors in the nucleus accumbens or ventral tegmental area; this is not the case for the corticotropin-releasing factor $CRF_2$ antagonist, which only blocks peripheral receptors such as those located in the pituitary. These different profiles of blockade should be considered when interpreting the results.

## 5. Conclusions

In conclusion, our results confirm that central corticotropin-releasing factor $CRF_1$ receptors and corticotropin-releasing factor $CRF_2$ receptors in areas no protected by blood brain barrier, like pituitary, differentially modulate the effects of social stress on reward and locomotion induced by cocaine. Therefore, these receptors should be considered potential therapeutic targets for evaluation by research about the treatment of addiction.

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# Study 2

Indomethacin blocks the increased conditioned rewarding effects of cocaine induced by repeated social defeat.

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RESEARCH ARTICLE

# Indomethacin blocks the increased conditioned rewarding effects of cocaine induced by repeated social defeat

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## Abstract

It is well established that repeated social defeat stress can induce negative long-term consequences such as increased anxiety-like behavior and enhances the reinforcing effect of psychostimulants in rodents. In the current study, we evaluated how the immune system may play a role in these long-term effects of stress. A total of 148 OF1 mice were divided into different experimental groups according to stress condition (exploration or social defeat) and pre-treatment (saline, 5 or 10 mg/kg of the anti-inflammatory indomethacin) before each social defeat or exploration episode. Three weeks after the last social defeat, anxiety was evaluated using an elevated plus maze paradigm. After this test, conditioned place preference (CPP) was induced by a subthreshold dose of cocaine (1 mg/kg). Biological samples were taken four hours after the first and the fourth social defeat, 3 weeks after the last defeat episode, and after the CPP procedure. Plasma and brain tissue (prefrontal cortex, striatum and hippocampus) were used to determine the levels of the pro-inflammatory cytokine interleukin 6 (IL-6). Results showed an increase of peripheral and brain IL-6 levels after the first and fourth social defeat that was reverted three weeks later. Intraperitoneal administration of the anti-inflammatory drug indomethacin before each episode of stress prevented this enhancement of IL-6 levels and also reversed the increase in the rewarding effects of cocaine in defeated mice. Conversely, this protective effect was not observed with respect to the anxiogenic consequences of social stress. Our results confirm the hypothesis of a modulatory proinflammatory contribution to stress-induced vulnerability to drug abuse disorders and highlight anti-inflammatory interventions as a potential therapeutic tool to treat stress-related addiction disorders.

## Introduction

Scientific evidence suggests that alterations of inflammatory parameters are linked to a vulnerability to mental illnesses [1], including depression [2, 3, 4], bipolar disorder [5, 6, 7], schizophrenia [8, 9, 10] and autism [11]. In addition, substance use disorders (SUD) are assumed to be related to changes in the immune system activity [12, 13]. Addiction can be considered a

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multifactorial mental disorder caused by an interaction between biological and environmental factors [14, 15, 16]. Although the exact mechanisms of the genesis of addiction have not been completely unraveled, a growing body of evidence relates alterations of the immune response to a possible cause of vulnerability to SUD [17, 18]. Neuroinflammation could help to explain some of the effects of drugs, including toxicity, deleterious cognitive effects [17, 19, 20, 21] and reward modulation, particularly taking into account that the immune signal significantly modulates the mesolimbic dopamine system [18, 22].

Both clinical and preclinical studies have shown that psychostimulants such as cocaine or methamphetamine activate the central and peripheral components of the innate immune system [12, 23, 24, 25, 26]. Repeated consumption of psychostimulants promotes a neuroinflammatory pattern characterized by an enhanced activation of glial and microglial cells [12] and increased release of glial cytokines with potential neurotoxic effects [27]. Moreover, plasmatic cytokines levels are under consideration by some researchers as possible biomarkers for cocaine users [24, 28].

Another cornerstone of our understanding of drug addiction is stress, with emotional stressors representing the main source of stress in humans [29]. Life-threatening situations induce a physiological response to stress which is adaptive and crucial for survival, and a failure to end that response may induce deleterious effects [30, 31]. In pre-clinical research, social defeat in an agonistic encounter is a rodent model with ecological validity that mimics real-life situations of social stress [32, 33, 34]. It has repeatedly been reported that different models of social defeat stress enhance the unconditioned and conditioned rewarding response to psychostimulant drugs and precipitate the reinstatement of drug seeking in the self-administration and conditioned place preference (CPP) paradigms [29, 34, 35, 36]. Alterations in the corticotrophin-releasing factor (CRF) neurotransmission system [35, 37, 38], epigenetic forms of plasticity [39] or inflammatory processes appear to be central to stress-induced vulnerability [4, 40, 41].

Recent basic research shows that stress induces activation of the immune system, thereby promoting the stimulation of microglia and leukocytes, altering the levels of peripheral and brain cytokines and leading to monocytes trafficking into the brain [40, 42]. Moreover, pro-inflammatory markers alter the permeability of the blood brain barrier (BBB) [43], which is also affected by social stress procedures [44]. When the BBB is compromised, peripheral immune cells can penetrate the central nervous system (CNS), thus causing or enhancing existing neuro-inflammation [45]. In this way, inflammatory processes are being posited as a link between stress and disease, by altering behavioral and neuroendocrine functions and leading to the vulnerability and enhanced sensibility to drugs that is reported after social stress procedures.

In response to this link between immune response, vulnerability to addiction and stress, some researchers have employed anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) as a therapeutic approach. For instance, the inflammatory potential of ethanol is well established [18, 20, 21, 46, 47]; it produces an inflammatory response via activation of microglia and astrocytes, contributing to neurodegeneration and the deleterious effects reported in alcoholics [48, 49] and in animal studies [20, 21, 50]. Pascual and coworkers [46] found that administration of indomethacin prior to ethanol binge-drinking prevented ethanol-induced brain damage in adolescent rats, as it blocked neural cell death and also attenuated short and long-term detrimental effects on cognitive and motor processes. Other researchers found that anti-inflammatory treatments also reverse cognitive impairments induced by social stress in animal models [42, 51].

Based on the above-mentioned observations, we hypothesize that the long-term sensitization to the rewarding properties of cocaine, constantly reported in experimental animals after

social defeat stress, is mediated by a pro-inflammatory state. Likewise, we hypothesize that this inflammatory mechanism is also underlying the long-term anxiety-like behavior displayed by social defeated animals. In order to test these hypotheses, we first determined whether there is an increase in peripheral and brain inflammatory response after social stress by measuring plasmatic and brain levels of interleukin 6 (IL-6). We then determined if anti-inflammatory treatment with indomethacin before each stress episode blocks the inflammatory response induced by social stress, and therefore the enhanced cocaine response and anxiety-like behavior expected after social stress experiences.

## Material and methods

### Animals

A total number of 148 OF1 adult mice (Charles River, France) were used in this study. The animals were housed as previously described in detail [35] in groups of four in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. To reduce their stress levels in response to experimental manipulations, mice were handled for 5 minutes per day on each of the 3 days prior to initiation of social defeat experiences. Aggressive opponents were individually housed in plastic cages (21 × 32 × 20 cm) for a month prior to initiation of the experiments in order to heighten aggression [52]. All mice were housed under the following conditions: constant temperature; a reversed light schedule (white light on 8:00–20:00 hours); and food and water available *ad libitum*, except during behavioral tests. The experimental protocol has been approved by an Institutional Review Committee for the use of animal subjects (Comité d'Ètica d'Experimentació i Benestar Animal, number A1426847710979). 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. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Drugs

The anti-inflammatory indomethacin (Sigma-Aldrich, Spain) was dissolved in 5% DMSO (dimethyl sulfoxide) and injected intraperitoneally (i.p.) at a dose of 5 or 10 mg/kg 30 minutes before each social defeat. For CPP, a dose of 1 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Spain) was employed. This dose of cocaine was selected on the basis of previous CPP studies showing 1 mg/kg to be a sub-threshold dose [53, 54, 55]. All the treatments were adjusted to a volume of 0.01 ml/g of weight. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drugs.

### Experimental design

The experimental design is depicted in Table 1. We studied whether social stress could induce an inflammatory response and if the anti-inflammatory indomethacin could modulate the effects of social defeat on the conditioned rewarding effects of cocaine (1 mg/kg). Mice received physiological saline, or a 5 or 10 mg/kg dose of indomethacin prior to each social defeat (RSD) (RSD-SAL, RSD-INDO5, RSD-INDO10) or exploration (EXP) (EXP-SAL, EXP-INDO10). Subsequently, 19 days after the last social defeat, anxiety was evaluated in the elevated plus maze (EPM) test. One day afterwards, the CPP procedure was initiated.

Biological samples were taken four hours after the first and fourth social defeats, 3 weeks after the last social defeat, and following the CPP procedure. In the case of the control group (EXP), samples were also taken four hours after the first exploration session and after the CPP procedure.

**Table 1. Experimental design.**

| | RSD / Exploration | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1st** | **2nd** | **3rd** | **4th** | | | |
| **1st set of mice** | SAL/ INDO5 / INDO 10 | SAL/ INDO5 / INDO 10 | SAL/ INDO5 / INDO 10 | SAL/ INDO5 / INDO 10 | EPM | 1 mg/kg cocaine CPP | Brain extraction and Blood samples |
| **2nd set of mice** | Brain extraction and Blood samples | | | Brain extraction and Blood samples | Brain extraction and Blood samples | | |
| **Experimental day** | **1** | **4** | **7** | **10** | **29** | **31–40** | **60–70** |

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## Apparatus and procedures

**Procedure of social defeat.** The social defeat protocol performed in this study was validated and described in detail in previous research papers from our research group [35, 36, 44]. Each of the social defeat episodes consisted of three phases, each of which began by introducing the "intruder" (the experimental animal) into the home cage of the "resident" (the aggressive opponent) for 10 minutes [56]. During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus leading to instigation and provocation [57]. The wire mesh was then removed from the cage to allow confrontation between the two animals for a 5-minute period. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 minutes to allow for social threats by the resident. Intruder mice were exposed to a different aggressor 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 [52]. In order to minimize the physical wounding during social defeats, the 5-minute direct encounters were finished earlier in the intruder displayed submissive supine posture for more than 8 seconds or if it was bitten by the aggressor more than 12 times. All agonistic encounters were videotaped to confirm social defeat.

**Conditioned place preference-CPP.** Place conditioning consisted of three phases and took place during the dark cycle [58], following an unbiased procedure in initial spontaneous preference terms as previously described in detail [35, 59]. For place conditioning, twelve identical Plexiglas boxes with black and white equal-sized compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm) were used. The CPP protocol consists of three phases, the first one being the pre-conditioning (Pre-C). In Pre-C phase, mice were given access to both compartments of the CPP box for 15 min (900 s) for 3 consecutive days. For the evaluation of the initial/natural preference, we use the data for the time spent by the animal in each compartment registered during the third (last) day of the pretest. Animals showing a strong, unconditioned aversion (less than 33% of the session time, i.e., 300 s) or preference (more than 67%, i.e., 600 s) for any compartment were excluded for the experiment. After this initial analysis of the natural preferences, a CPP box compartment (black or white) was chosen to be paired with the drug and the other one with the vehicle, taking into account that, in each group, half the animals received the treatment in the most preferred compartment and the other half in the least preferred one. Additionally, we statistically confirmed that there were no significant differences between the time spent in the drug-paired and the saline-paired compartments to avoid any preference bias before conditioning.

In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 minutes. After a 4-hour interval, they received cocaine immediately before

being confined to the drug-paired compartment for 30 minutes. In the third phase or post-conditioning (Post-C), the time spent by the untreated mice in each compartment during a 15-minute observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the induction of an aversion.

All groups in which a preference for the drug-paired compartment was established underwent an extinction session every 72 hours, which consisted of placing the mice in the apparatus for 15 minutes. This was repeated until the time spent in the drug-paired compartment by each group was similar to that of the Pre-C.

The effects of non-contingent administration of a priming dose of cocaine were evaluated 24 hours after the confirmation of extinction. Reinstatement tests were the same as those for the Post-C (free ambulation for 15 minutes), except for the fact that mice were tested 15 minutes after administration of the drug (half of the dose used for conditioning). This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

**Elevated plus maze-EPM.** The elevated plus maze (EPM) test was carried out essentially following the procedure previously described by Daza-Losada and coworkers [59]. The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 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. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 hour prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 minutes. The maze was thoroughly cleaned with a damp cloth after each trial. The measurements recorded during the test period were number of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. The time and percentage of time spent in the open arms and the number of open arm entries are generally used to characterize the anxiolytic effects of drugs. In addition, the number of closed and total entries indicates motor activity.

## Tissue sampling

To obtain blood and tissue samples, unperfused mice were sacrificed by cervical dislocation and then decapitated. Blood was collected from the neck into a Microvette CB 300 capillary tube (Sarstedt, Germany). Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 minutes, 5000G) and transferred to sterile 0.2 ml microcentrifugue tubes. Plasma samples were stored at -80˚C until IL-6 concentration determination.

Brains were rapidly removed and the prefrontal cortex (PFC), striatum (STR) and hippocampus (HIP) were dissected following the procedure described by Heffner and coworkers [60] and kept on dry ice until storage at -80˚C. Prior to IL-6 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches and coworkers [61]. Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM $Na_3VO_4$, and 10 mM PMSF). Brain homogenates were kept on ice for 30 minutes and centrifuged at maximum speed for 15 minutes, after

which the supernatant was collected and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

### IL-6 ELISA assay

To determine IL-6 concentration in plasma and tissues, we used a Mouse IL-6 ELISA Kit obtained from Abcam (Ref: ab100712) following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450nm and the final results were calculated using a standard curve following the manufacturer's instructions, and were expressed as pg/ml for plasma, and pg/mg for tissue samples.

### Statistical analyses

For the CPP data, the time spent in the drug-paired compartment during Pre-C and Post-C tests was analyzed with a mixed three-way ANOVA, with two between-subjects variables—Pre-treatment, with three levels (Saline, Indomethacin 5 or 10 mg/kg), and Stress, with two levels (RSD and EXP)—and a within-subjects variable—Days, with two levels (Pre-C and Post-C). For the EPM data, a two-way ANOVA, with two between-subjects variables—Pre-treatment, with two levels (Saline or Indomethacin 10), and Stress, with two levels (RSD and EXP)—was employed. In all cases, post-hoc comparisons were performed with Bonferroni tests. In addition, the groups showing CPP, extinction and reinstatement values were analyzed by a Student's t-test.

Data concerning IL-6 concentration were analyzed using a single factor analysis ANOVA, with a between-subjects variable: Stress, with 4 levels (Exploration, first social defeat, fourth social defeat and 3 weeks after the last social defeat). For the IL-6 levels measure performed after the CPP procedure, we used an ANOVA with a between-subjects variable: Stress, with 3 levels (Exploration, RSD and RSD plus Indomethacin). Data are presented as mean ± SEM. A p-value $< 0.05$ was considered statistically significant. Analyses were performed using SPSS v22.

## Results

### Indomethacin blocks the increase in the conditioned rewarding effects of cocaine (1 mg/kg) induced by social defeat stress

ANOVA of the CPP data (Fig 1) showed a significant effect of the variable Days $F(1,68) = 10.905$, $p < 0.01$ and the interactions Days x Pre-treatment $F(2,68) = 4.657$, $p < 0.05$ and Days x Pre-treatment x Stress $F(1,68) = 3.862$, $p < 0.05$. As expected, socially defeated animals pre-treated with saline (RSD-SAL) developed CPP, since they spent more time in the drug-paired compartment in the Post-C than in the Pre-C test ($p < 0.05$), a preference that was not observed in defeated mice treated with the highest dose of indomethacin (RSD-INDO10), in contrast with the CPP developed in defeated mice treated with the lowest dose of indomethacin (RSD-INDO5 $p < 0.001$). Neither of the groups that developed preference for the drug-paired compartment showed reinstatement after a priming dose of 0.5 mg/kg cocaine.

**Indomethacin fails to prevent the long-term anxiogenic effects of social defeat.** The data of the EPM test are presented in Table 2. ANOVA for the time spent in the open arms $F(1,59) = 18.360$, $p < 0.001$, the percentage of time spent in the open arms $F(1,59) = 15.751$, $p < 0.001$, the number of entries into the open arms $F(1,59) = 4.594$, $p < 0.05$, the percentage of entries into the open arms $F(1,59) = 15.793$, $p < 0.001$, the time spent in the closed arms $F(1,59) = 5.267$, $p < 0.05$, the number of entries into the closed arms $F(1,59) = 19.641$,

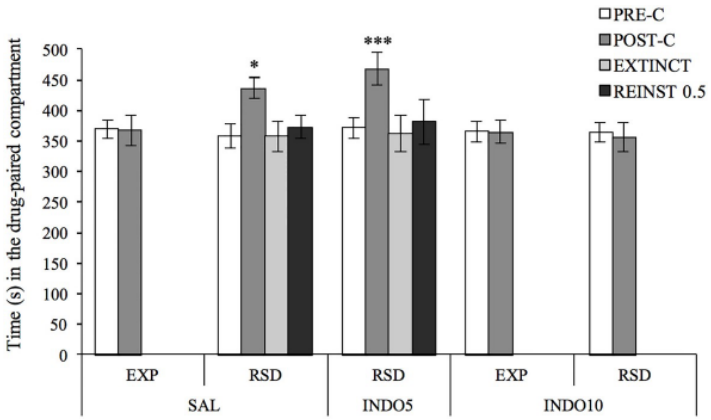


**Fig 1. Administration of the highest indomethacin dose before each social defeat blocked acquisition of the CPP induced by 1 mg/kg of cocaine in defeated mice.** Before the social stress protocol animals were randomly assigned to the following groups according to the pre-treatment they received: saline (EXP-SAL n = 13; RSD-SAL n = 14); 5 (RSD-INDO5 n = 16) or 10 mg/kg (EXP-INDO10 n = 13; RSD-INDO10 n = 17) of indomethacin. Bars represent the time (s) spent in the drug-paired compartment before conditioning sessions in the PRE-C test (white bars), after conditioning sessions in the POST-C test (dark grey bars), in the last extinction (EXTINCT) session (light gray bars), and in the reinstatement (REINST 0.5) test (black bars). Data presented as mean values ± SEM $^{*}p < 0.05$, $^{***}p < 0.001$ significant difference in the time spent in the drug-paired compartment versus PRE-C.

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$p < 0.001$ and the total number of entries $F(1,59) = 19.887$, $p < 0.001$ revealed a significant effect of the variable Stress. Post-hoc analyses showed that socially defeated animals spent less time, a lower percentage of time and a lower percentage of entries into the open arms ($p < 0.001$ in all cases), while they spent more time in the closed arms ($p < 0.05$), made more total entries ($p < 0.001$), and more entries into the open ($p < 0.05$) and closed arms ($p < 0.001$) than control non-stressed animals.

## Social defeat increases brain and peripheral IL-6 levels

Regarding plasmatic circulating IL-6 levels (Fig 2a), the ANOVAF $F(3,37) = 10.321$, $p < 0.001$ showed that levels were significantly increased in defeated mice after the first ($p < 0.001$) and the fourth ($p < 0.01$) social defeat compared with non-stressed animals. However, no differences were detected in plasma three weeks after the final exposure to stress.

**Table 2. Administration of indomethacin before each SD fails to prevent the long-lasting anxiogenic effect of stress in the EPM.**

| | EXP | | RSD | | |
|---|---|---|---|---|---|
| | SAL | INDO10 | SAL | INDO5 | INDO10 |
| **Time OA (s)** | 88 ± 9 | 96 ± 13 | 60 ± 7 * | 56 ± 12 * | 47 ± 7 * |
| **% time OA** | 38 ± 3 | 37 ± 5 | 25 ± 3 * | 24 ± 5 * | 20 ± 3 * |
| **Open entries** | 17 ± 4 | 23 ± 4 | 33 ± 6 *** | 31.3 ± 6 *** | 26 ± 4 *** |
| **% entries OA** | 39 ± 4 | 45 ± 2 | 29 ± 4 * | 28 ± 5 * | 26 ± 4 * |
| **Time in CA (s)** | 146 ± 10 | 172 ± 18 | 181 ± 9 *** | 186 ± 17 *** | 193 ± 10 *** |
| **Closed entries** | 28 ± 6 | 25 ± 2 | 86 ± 17 * | 89 ± 21 * | 80 ± 14 * |
| **Total entries** | 45 ± 9 | 48 ± 5 | 119 ± 20 * | 120 ± 24 * | 105 ± 16 * |

Data are presented as mean values ±S.E.M.

*p <0.05;

***p <0.001 significant difference with the exploration groups.

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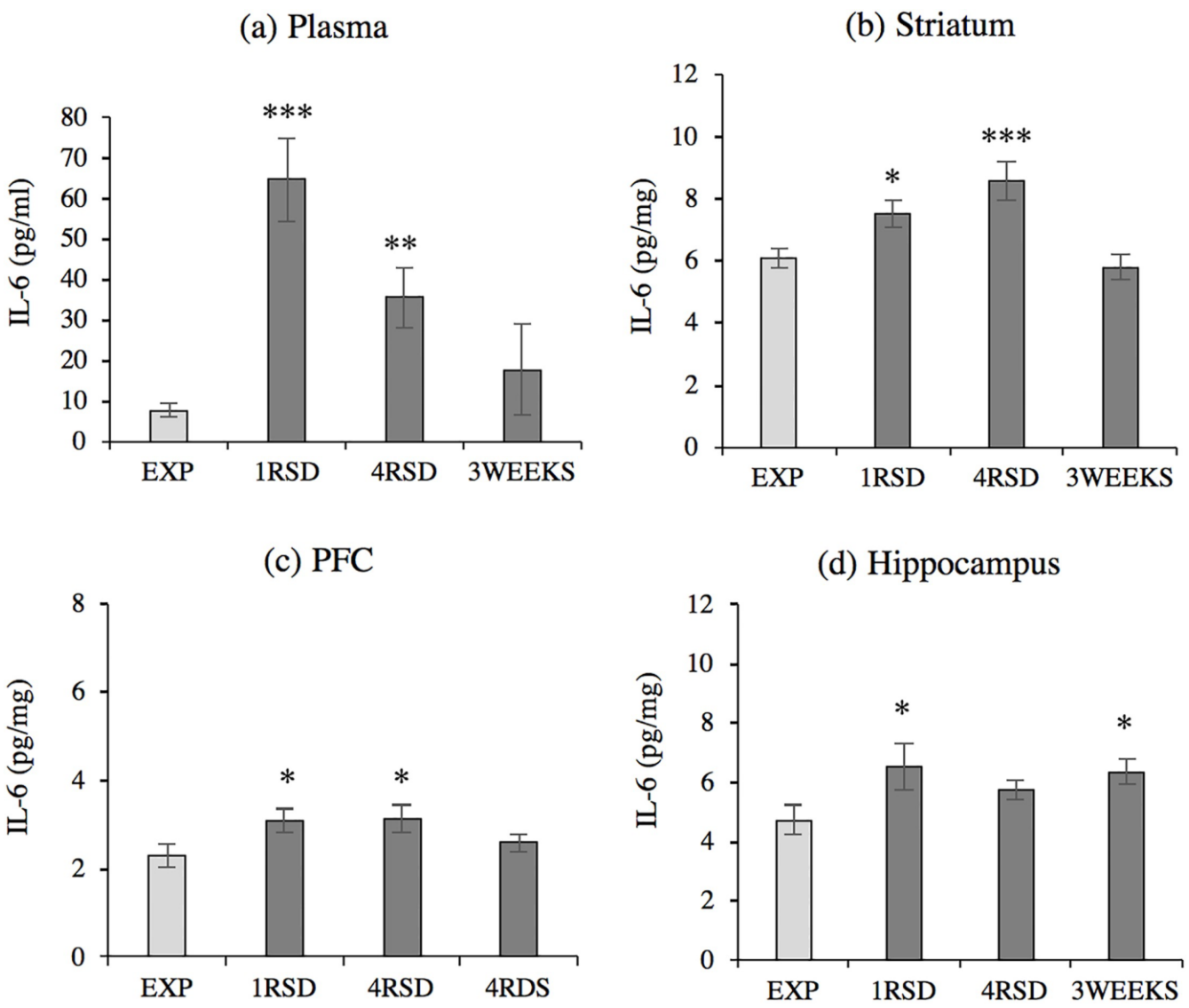


**Fig 2. Social defeat increases IL-6 levels in the plasma, STR, PFC and hippocampus.** Groups defined by stress condition and social defeat episode: (a) Plasma (EXP n = 11, 1RSD n = 11, 4RSD n = 11, 3WEEKS n = 8); (b) Striatum (EXP n = 12, 1RSD n = 12, 4RSD n = 12; 3WEEKS n = 8); (c) PFC (EXP n = 7, 1RSD n = 7, 4RSD n = 7; 3WEEKS n = 7); (d) Hippocampus (EXP n = 8, 1RSD n = 8, 4RSD n = 8; 3WEEKS n = 8). Data are presented as mean values ± SEM (pg/ml in plasma and pg/mg in brain tissue) $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. exploration (EXP) group.

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Similar results were observed in the three brain areas studied. The ANOVA revealed a significant increase in IL-6 protein levels in the STR $F(3,40) = 7.878$, $p < 0.001$ (Fig 2b); PFC $F(3,24) = 2.991$, $p < 0.05$ (Fig 2c) and hippocampus $F(3,28) = 2.891$, $p < 0.05$ (Fig 2d) after the first social defeat ($p_s < 0.05$), in the STR ($p < 0.001$) and the PFC ($p < 0.05$) after the fourth defeat, and only in the hippocampus three weeks after the last social defeat ($p < 0.05$).

## Indomethacin blocks the increase induced by social defeat in IL-6 levels

The ANOVA $F(3,27) = 2.971$, $p < 0.05$ of plasmatic IL-6 levels in animals pretreated with indomethacin before social stress (Fig 3) showed that plasmatic IL-6 protein levels were

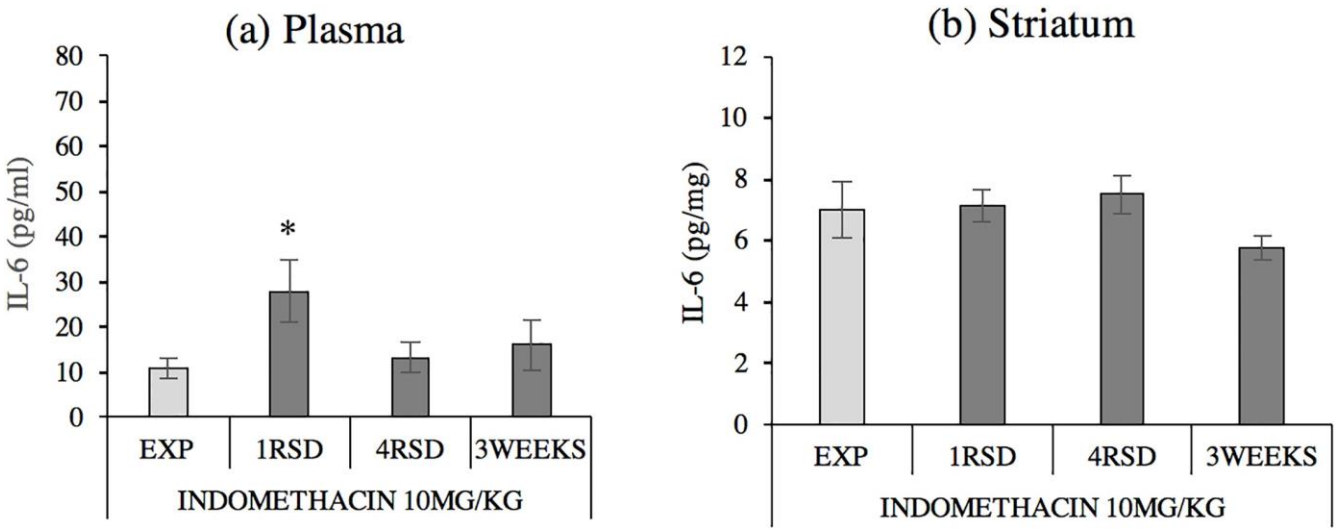


**Fig 3. Indomethacin decreases plasma and striatum (STR) IL-6 levels in defeated mice.** Groups defined by stress condition and social defeat episode, all pretreated with indomethacin 10 mg/kg: (a) Plasma (EXP n = 7, 1RSD n = 8, 4RSD n = 8, 3WEEKS = 8); (b) Striatum (EXP n = 6, 1RSD n = 7, 4RSD n = 8, 3WEEKS n = 8). Data are presented as mean values ± SEM (pg/mg) $^{*}p < 0.05$ vs. exploration (EXP) group.

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significantly increased with respect to controls in defeated mice only after the first social defeat ($p < 0.05$). No differences were detected in the STR $F(3,25) = 1.969$, $p > 0.05$.

## Increased plasma and brain IL-6 levels after cocaine-induced CPP in defeated mice

The ANOVA $F(2,21) = 19.279$, $p < 0.001$ of plasmatic IL-6 levels after cocaine CPP (Fig 4) revealed significantly increased IL-6 protein levels in defeated mice compared to the

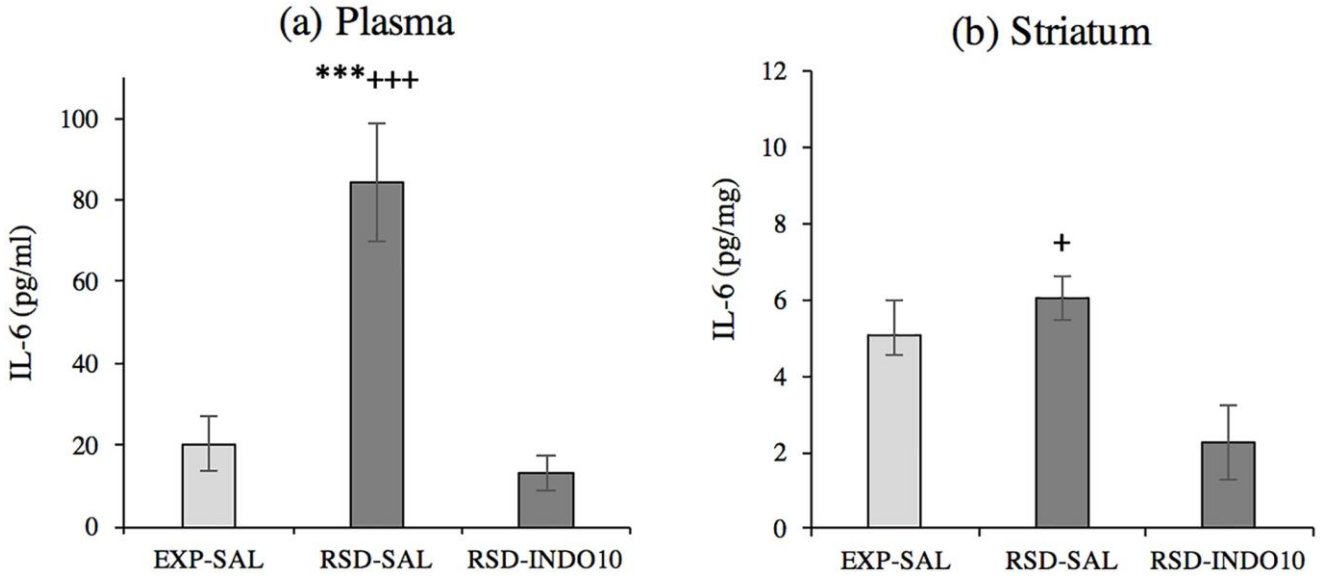


**Fig 4. Social defeat increases plasma and striatal IL-6 levels after cocaine-induced CPP.** Groups defined by stress condition and pre-treatment: (a) Plasma (EXP-SAL n = 8, RSD-SAL n = 8, RSD-INDO10 n = 8); (b) Striatum (EXP-SAL n = 6, RSD-SAL n = 6, RSD-INDO10 n = 10). Data are presented as mean values ± SEM (pg/mg); $^{***}p < 0.001$ vs. exploration (EXP) group.; $+++p < 0.001$; $+p < 0.05$ vs. pretreated defeated (RSD-INDO10) group.

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exploration group ($p < 0.001$). Pre-treatment with indomethacin before each social stress episode prevented this increase, as no significant differences were detected in plasmatic levels of socially defeated animals pretreated with indomethacin versus non-stressed controls, while a significant difference was observed when compared to socially defeated animals without anti-inflammatory treatment ($p < 0.001$).

Similar results were obtained in the STR, as ANOVA $F(2,19) = 5.644$, $p < 0.01$ showed increases in IL-6 protein levels in defeated animals after CPP when compared with stressed mice previously treated with indomethacin ($p < 0.05$).

## Discussion

In the current study, we explored the role of the immune system in the long-term effects of social stress on anxiety-like behavior and the conditioned rewarding effects of cocaine in mice. Social defeat induced a long-term increase in anxiety when evaluated with the EPM test and produced a significant increase in the conditioned reinforcing effect of cocaine in the CPP paradigm. With the aim of determining a possible role of the immune response in the genesis of these stress effects, we first verified that social defeat increased levels of the proinflammatory cytokine IL-6. Pre-treatment with the anti-inflammatory drug indomethacin before each stress episode prevented this enhancement of IL-6 levels and reversed the increase in the rewarding effects of cocaine in defeated mice. Conversely, this protective effect was not observed with respect to the anxiogenic consequences of social stress.

It has been widely demonstrated, in humans and animal models, that stressful experiences have a modulatory effect on the behavioral and physiological response to drugs [62]. In general, social stress induces a sensitization of the reward system that makes mice more sensitive to the effects of drugs [63]. In this regard, the CPP paradigm is a useful tool to study how stress can modify sensitivity to the secondary motivational properties and hedonic valence of drugs [64, 65]. In the present experiments, we observed that socially defeated animals developed CPP with a sub-threshold dose of cocaine (1 mg/kg), while this conditioned preference was not observed in animals under the exploration condition (non-stressed). These results are in agreement with previous data reported by our group for cocaine [35, 36, 44] and other substances such as alcohol or MDMA [66]. We also found that defeated animals displayed a long-term increase in anxiety-like behavior, spending less time and a lower percentage of time, and performing fewer entries and a lower percentage of entries into the open arms of the EPM than their non-stressed counterparts. This result has also been consistently replicated in the literature [66, 67, 68, 69, 70, 71].

We hypothesized that these behavioral consequences of social stress are somehow mediated by a neuroinflammatory immune response. To validate this hypothesis, we first determined if social stress could trigger an inflammatory response. We observed increased levels of the cytokine IL-6 in defeated mice four hours after social defeat episodes. Socially defeated animals displayed significantly higher plasmatic and brain (STR, PFC and hippocampus) IL-6 levels after the first and fourth social defeat when compared with exploration mice. This is not surprising, as other researchers have also reported increased levels of proinflammatory cytokines in response to social stressors [40, 68, 72]. For example, Hodes and coworkers [40] found that the higher responsiveness of the immune system to stress—characterized by higher levels of pro-inflammatory cytokines—was correlated with a higher vulnerability of mice to a stress-induced depressive-like phenotype.

However, most of these previous reports only dealt with the acute inflammatory consequences of social stress. We have focused on long term-effects in the present study by extending the timeframe of the IL-6 profile and determining its levels three weeks after the stress

episode, immediately before performing the behavioral tests. Three weeks after the last social defeat encounter a significant increase in IL-6 levels was registered only in the hippocampus of socially defeated mice, which is considered a central structure in CPP establishment [73, 74]. After continuous exposure to the aggressor stimulus for 10 days, other researchers have reported an up-regulated plasmatic IL-6 levels up to 35 days after the last defeat episode [40]. We believe that these discrepancies may be a result of our shorter and intermittent social stress protocol, while the other model can be considered chronic. It should be stressed that social defeat involves physical contact during the aggressive encounter and can sometimes incur physical wounding as a consequence, which can confound the interpretation of the inflammatory measures in the brain or the blood. While some researchers did not report alterations in inflammatory markers after RSD when physical wounding is completely suppressed [75], other investigators find alterations in cytokines and in the immune response after non-physical social stress models, such as social threat exposure in juvenile mice [76] or vicarious social defeat in adults [40, 77], findings that corroborate the immune response to social stressors. We are aware of the possible interference of physical injuring in the immune response and, therefore, our protocol of RSD has been designed to minimize the physical wounding following the indications of Burke and co-workers [78].

Once we confirmed the existence of an acute immune reaction triggered by social stress episodes, we aimed to determine if the increased sensitivity to the rewarding properties of cocaine and anxiety-like behavior is somehow modulated by this pro-inflammatory response. Considering that cytokine IL-6 levels were generally similar in stressed and non-stressed mice (with the exception of the hippocampus) when they performed the anxiety and CPP tests, the different behavior of defeated mice can be explained by an initial role of the pro-inflammatory response by which long-term adaptations are promoted. For this reason, we decided to block the development of an inflammatory response by administering the anti-inflammatory indomethacin before each social stress episode. At the highest dose of indomethacin (10 mg/kg), we registered a general assuaging of the increase in IL-6 levels after the first and fourth social defeat compared with levels displayed in non-treated animals, although significantly higher levels of IL-6 in plasma continued to be measured after the first defeat when compared with the exploration group.

We also detected increased levels of IL-6 after the CPP procedure. Administration of four daily low doses of cocaine (1mg/kg) induced an enhancement of plasmatic IL-6 cytokine levels in all animals. This enhanced immune signaling was more pronounced in animals under the stress condition. Socially defeated animals presented increased IL-6 levels in plasma, with these levels proving to be statistically higher than in non-stressed animals. Again, pretreatment with indomethacin reversed this enhancement in the effect of stress, and socially defeated animals pretreated with the anti-inflammatory displayed similar IL-6 levels to the exploration group after cocaine CPP. The potential of cocaine as a xenobiotic that can activate proinflammatory central immune signaling is well documented [See revisions 17, 19]. We have found that the inflammatory potential of cocaine is exacerbated by previous stress experience, whereas an anti-inflammatory pre-treatment before stress can reverse it. It is known that previous stress experiences can sensitize peripheral and central components of the immune system [68, 69, 70]. We hypothesized that our social stress paradigm would induce long-term changes in the immune response of our experimental mice, making their immune system more reactive to insults. Indeed, indomethacin administration before each social defeat blocked the proinflammatory response induced by social stress and avoided the development of sensitization of the neuroimmune axis.

Once we had demonstrated that indomethacin was capable of reducing the release of cytokine induced by social defeat, we set out to evaluate if this decrease was related to the

behavioral consequences of stress. Administration of the higher dose of indomethacin (10 mg/kg) before each social defeat completely reversed the stress-induced increase in the rewarding properties of cocaine, since defeated mice treated with this anti-inflammatory did not develop CPP induced by cocaine. One possible mechanism by which neuroinflammation can enhance the rewarding properties of cocaine is the activation of the hypothalamus hypothalamic-pituitary-adrenal (HPA) axis. It had been demonstrated that IL-6 promotes the activation the HPA axis at the hypothalamic level [79, 80], leading to the release of CRF. CRF modulates dopamine function due to its interaction within the ventral tegmental area, a key structure for drug reward effects [81, 82]. Elevated CRF levels would alter the function of the dopaminergic neurons, causing long-term neuroadaptations along this pathway [83, 84]. In this sense, increased levels of CRF in the ventral tegmental area have recently been related to increased rewarding properties of cocaine in the self-administration paradigm [85, 86].

Conversely, the anti-inflammatory treatment failed to prevent anxiety-like behavior in our socially defeated animals. While there is general agreement in the literature about the link between an enhancement of IL-6 levels and the induction of anxiety (See revision on [87]), the decrease of IL-6 levels was not effective in blocking the anxiogenic consequences of social defeat in our study. Hodes and collaborators [40] reported similar results in an experiment carried out with socially stressed mice pretreated with an IL-6 monoclonal antibody (mAbs), a pharmacological intervention that neutralizes circulating IL-6 cytokine, thereby preventing it from binding to IL-6 receptors in cell membranes. The authors found that chronic administration of IL-6 mAb prevented the development of social avoidance, which they considered a marker of susceptibility to stress consequences, while it failed to reduce anxiety-like behavior induced by stress in the EPM. One possible explanation of this discrepancy between the depressive and anxiogenic consequences of social stress may be different mechanisms for their genesis. Regarding this, Wohleb and collaborators [68, 69, 70] proposed a neuroinflammatory mechanism for the genesis of anxiety that is in line with our present results. They theorized that the pro-inflammatory profile induced by stress alters the function of vascular endothelial cells in the BBB, allowing peripheral monocytes to traffic into the brain, leading to the development of anxiety. They confirmed this hypothesis using transgenic knockdown mice for the proinflammatory interleukin-1 receptor (IL-1R1) in endothelial cells. These transgenic mice are protected from increased permeability of the inflammatory signals between brain resident microglia and peripheral blood monocytes, and, as a result, have a decreased central inflammation and did not develop anxiety-like behavior after social stress when compared with wild type mice [70]. Additionally, Wohleb and collaborators reported that this monocyte infiltration into the brain occurs in regions specifically associated with fear, anxiety and threat appraisal such as the prefrontal cortex, the hippocampus, or the amygdala while it is not registered in other regions such as the motor or somatosensory cortex, and the STR [88]. This region specificity of monocyte infiltration could explain why indomethacin failed to block the genesis of anxiety while it was effective in blocking the stress-induced increase in the rewarding properties of cocaine. As the indomethacin treatment attenuated but did not completely block the increase of peripheral IL-6 after the first RSD, we hypothesize that this increase was enough to start the mechanism that led to increases in the permeability to BBB. The decreased integrity of the BBB let monocytes traffic into the more permeable brain regions, while other regions less susceptible for this peripheral infiltration, such as the striatum (key in reward drug response), were not affected.

## Conclusions

The results of this research surpass current basic knowledge and take on a clear translational relevance, since IL-6 levels have been found to be altered in humans under conditions of social

stress [83], and even more so in those with cocaine use disorders [17]. The confirmation of the contribution of inflammation to stress-induced vulnerability to mental-disorders provides new research opportunities, especially in the field of drug abuse disorders; from considering inflammatory parameters as a possible biomarker for diagnosis, to developing anti-inflammatory strategies as preventive or therapeutic interventions.

## Supporting information

**S1 File. CPP experimental data.**
(XLSX)

**S2 File. EPM experimental data.**
(XLSX)

**S3 File. IL-6 concentration data.**
(XLSX)

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# Study 3

Oxytocin prevents the increase of cocaine-related responses produced by social defeat.

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# Oxytocin prevents the increase of cocaine-related responses produced by social defeat

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## HIGHLIGHTS

- Social defeat stress induces a long-lasting increase in anxiety like behavior.
- Social defeat stress enhances rewarding properties of cocaine.
- Social defeat stress increase BDNF levels in prefrontal cortex.
- Oxytocin treatment favors the extinction of cocaine-associated memories.
- Oxytocin administration counteracts stress effect in anxiety and in BDNF levels.

## ARTICLE INFO

*Keywords:*
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## ABSTRACT

The neuropeptide oxytocin (OXT) plays a critical role in the regulation of social and emotional behaviors. OXT plays a role in stress response and in drug reward, but to date no studies have evaluated its implication in the long-lasting increase of the motivational effects of cocaine induced by repeated social defeat (RSD). During the social defeat procedure, 1 mg/kg of OXT was administered 30 min before each episode of RSD. Three weeks after the last defeat, the effects of cocaine on the conditioned place preference (CPP), locomotor sensitization and the self-administration (SA) paradigms were evaluated. The influence of OXT on the levels of BDNF in the prefrontal cortex (PFC), striatum and hippocampus was also measured. Our results confirm that raising the levels of OXT during social defeat stress can block the long-lasting effects of this type of stress. OXT counteracts the anxiety induced by social defeat and modifies BDNF levels in all the structures we have studied. Moreover, OXT prevents RSD-induced increases in the motivational effects of cocaine. Administration of OXT before each social defeat blocked the social defeat-induced increment in the conditioned rewarding effects of cocaine in the CPP, favored the extinction of cocaine-associated memories in both the CPP and SA, and decreased reinstatement of cocaine-seeking behavior in the SA. In conclusion, the long-lasting effects of RSD are counteracted by administering OXT prior to stress, and changes in BDNF expression may underlie these protective effects.

## 1. Introduction

The neuropeptide oxytocin (OXT) plays a critical role in the regulation of social and emotional behaviors (Johnson and Young, 2017; Neumann and Slattery, 2016). OXT is a nine-amino acid cyclic neuropeptide synthesized mainly in the supraoptic and paraventricular nuclei (PVN) of the hypothalamus. It innervates brain regions associated with stress and reward, such as the amygdala, septum, nucleus accumbens (NAc) and the bed nucleus of stria terminalis, where OXT receptors (OXTR) are expressed (see Gimpl and Fahrenholz, 2001). OXT also plays an important role in learning and memory processes related to the fear response, and OXT neurons are activated by a variety of stressful

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stimuli (Onaka et al., 2012).

Several studies suggest that OXT produces prosocial behaviors, facilitating approach activities by inhibiting systems involved in social anxiety and defense (Heinrichs and Domes, 2008; Neumann et al., 2000). Generally, experiments involving administration of OXT reveal anxiolytic effects in humans (Kirsch, 2005; Petrovic et al., 2008) and animals (Neumann and Slattery, 2016; Smith and Wang, 2014; Yoshida et al., 2009), though anxiogenic reactions have also been described (Eckstein et al., 2014; Grillon et al., 2013; Peters et al., 2014), fueling the hypothesis that variables such as dose and administration context modulate the effects of OXT. On the other hand, it is well established that the OXT system is implicated in the homeostatic response to stress. Animal studies have highlighted profound alterations of said system after chronic administration of cocaine or following its withdrawal (Georgiou et al., 2016). In addition, postmortem studies in humans suffering from depression have revealed higher levels of OXT immunoreactive neurons in the hypothalamus (Purba et al., 1996). Altogether, these results indicate that OXT is a contributing factor to the pathophysiology of mood and drug-related disorders.

Stress is one of the main risk factors for depression, anxiety or addiction (Logrip et al., 2012; Sinha et al., 2011). The close association between the brain systems involved in the response to drugs of abuse and stress suggests environmental stressors can cause long-term changes in the brain's reward system function, inducing relapses in drug-seeking and -taking. The social defeat (SD) paradigm is considered the most representative animal model for studying the consequences of social stress (Hammels et al., 2015). The agonistic encounter between conspecifics of the same specie models the subordinate vs. outsider relation in human interactions (Selten et al., 2013; Tornatzky and Miczek, 1993). Numerous reports have proved that SD increases both cocaine self-administration (SA) and cocaine-induced conditioned place preference (CPP). Increased acquisition, motivation and reinstatement to take cocaine have been repeatedly observed using SA paradigms (Boyson et al., 2014; Covington and Miczek, 2001; Covington et al., 2005; Han et al., 2017; Holly et al., 2016; Quadros and Miczek, 2009). CPP studies have painted a similar picture, with SD inducing short- and long-lasting increases in the conditioned rewarding effects of cocaine, increasing the time needed to extinguish the preference and susceptibility to reinstatement of said preference (Hymel et al., 2014; McLaughlin et al., 2006; Montagud-Romero et al., 2016a and b; Land et al., 2009; Reguilón et al., 2017; Rodriguez-Arias et al., 2016, 2017).

SD stress induces pronounced physiological and endocrine responses, such as elevated levels of corticosterone (Lumley et al., 2000; Meerlo et al., 2002; Montagud-Romero et al., 2016b; Rodríguez-Arias et al., 2017), and alterations in the levels of BDNF (Wang et al., 2018; Xu et al., 2018). SD stress induces prolonged BDNF expression in the ventral tegmental area (VTA) (Berton et al., 2006; Fanous et al., 2010; Krishnan et al., 2007), and an enhanced expression of proBDNF in the dentate gyrus (DG) and basolateral amygdala (BLA) in adult and adolescent mice subjected to SD (Montagud-Romero et al., 2017). The diverse intracellular signaling pathways activated by BDNF may underlie responses to drugs, stress, or mood disorders (Nikulina et al., 2014). For example, increased BDNF levels within the mesolimbic dopamine (DA) system are associated with the development of a depressive-like phenotype (Nestler and Carlezon, 2006), and current evidence strongly implicates BDNF-TrkB signaling in the response to clinically used antidepressant drugs (for review see Björkholm and Monteggia, 2016). In addition, alterations in mesocorticolimbic BDNF expression have been associated with the abuse potential of many drugs (Fumagalli et al., 2007; Le Foll et al., 2005; McGough, 2004; Meredith et al., 2002; Numan et al., 1998).

SD stress activates the PVN OXT neurons and OXTR –expressing neurons in various brain regions, facilitating SD posture during defeat stress (Nasanbuyan et al., 2018). Recent research suggests that OXT modulates the stress response via its action on the hypothalamic–pituitary–adrenal (HPA) axis (Lukas and Neumann, 2014; Parker et al., 2005). The relation between OXT and SD on the other hand is not clear if current literature is consulted. Several studies indicate that SD does not alter OXT release (Engelmann et al., 1999; Wotjak et al., 1996). In line with this, Wang et al. (2018) observed that chronic SD reduced levels of OXT and OXTR in the shell region of the NAc. However, intra-NAc shell OXT microinjections reversed alterations in social behavior induced by chronic SD, whereas injections of an OXTR antagonist blocked these effects. Other studies have demonstrated that OXT expression in the PVN is enhanced after single social defeat or acute emotional stress (Ebner et al., 2000; Engelmann et al., 1999), and defeated mice have been shown to display elevated levels of OXTR mRNA levels in the lateral septum (Litvin et al., 2011). A recent report has described stress-induced increases in the activation OXTR and produces social withdrawal, while a single dose of OXT - either systemically or within the bed nucleus of the stria terminalis - reverses this stress-induced social avoidance in female mice (Duque-Wilckens et al., 2018). These findings imply an until now unappreciated therapeutic potential of OXT in stress-induced psychiatric disorders.

In line with this, there is strong evidence that OXT is a promising therapeutic agent for the treatment of addiction disorders. It has been hypothesized that there is an association between the oxytocinergic and dopaminergic systems by which they regulate motivational behaviors (Zanos et al., 2014). Central or peripheral administration of OXT acutely increases DA utilization within the NAc, while chronic administration of OXT decreases DA utilization within the basal forebrain of mice (Kovàcs et al., 1986, 1990). In addition, there is strong evidence that OXT plays a role in the rewarding effects of drugs of abuse. Studies in humans show that cocaine use decreases plasma OXT levels (Light et al., 2004), and OXT administration decreases stress-induced craving for marijuana (McRae-Clark et al., 2013) and alcohol withdrawal symptoms (Pedersen et al., 2013). Equally, in basic studies, OXT has been shown to reduce ethanol SA in mice (King et al., 2017; MacFadyen et al., 2016) and morphine tolerance and withdrawal effects (Sarnyai and Kovács, 2014). With respect to psychostimulants, OXT blocks methamphetamine-conditioned behaviors (Qi et al., 2009) and reduces reinstatement of methamphetamine seeking (Baracz and Cornish, 2016; Carson et al., 2010; Cox et al., 2013). Both central and peripheral OXT administration attenuates cocaine-induced locomotor hyperactivity, stereotypies (Kovàcs et al., 1990; Sarnyai et al., 1990, 1991) and tolerance to stereotypies (Sarnyai et al., 1992). Systemic OXT decreases cocaine intake during SA, and prime or cue-induced reinstatement of cocaine seeking (Leong et al., 2016, 2017; Sarnyai and Kovács, 1994; Zhou et al., 2014).

Despite the clear role of OXT in the stress response and in the rewarding effects of drugs, no studies to date have evaluated its role in the long-lasting increase of the motivational effects of cocaine induced by repeated social defeat (RSD). To respond to this gap in the literature, we administered 1 mg/kg of OXT 30 min before each episode of SD and three weeks after the last defeat, we evaluated the motivational effects of cocaine in different paradigms, including the CPP, the locomotor sensitization and the SA. Due to the pivotal role of BDNF in SD stress, we have also evaluated the effect of OXT on the levels of this neurotrophin in the prefrontal cortex (PFC), striatum (STR) and hippocampus (HIP) in socially defeated mice.

## 2. Material and methods

### 2.1. Animals

A total number of 240 OF1 adult mice (Charles River, France) were used in this study. Experimental mice were housed in groups of four in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. Aggressive opponents were individually housed in plastic cages (21 × 32 × 20 cm) for at least a month prior to the initiation of the experiments in order to heighten aggression (Rodríguez-Arias et al., 1998). All mice were housed under the following conditions: constant

temperature; a reversed light schedule (white light on 8:00–20:00 h); and food and water available *ad libitum*, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia and the UPF/PRBB respectively.

### 2.2. Drugs

For pre-treatment Oxytocin (Sigma-Aldrich, Spain) was dissolved in physiological saline (NaCl 0.9%) and injected intraperitoneally (i.p.) at a dose of 1 mg/kg 30 min before each social defeat episode. For CPP establishment, doses of 1 mg/kg and 10 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Spain) were used. These doses of cocaine were selected on the basis of previous CPP studies showing 1 mg/kg to be a non-effective dose and 10 mg/kg to be an effective dose (Arenas et al., 2014; Montagud-Romero et al., 2014; Vidal-Infer et al., 2012). For SA studies a non-effective dose of 0.5 mg/kg/infusion of cocaine was used for the acquisition phase and a priming injection of 10 mg/kg, i.p. to induce reinstatement. All i.p. administrations were adjusted in a volume of 0.01 ml/g of body weight. Saline control groups (SAL) were injected with physiological saline (NaCl 0.9%), which was also used to dissolve cocaine.

### 2.3. Experimental design

The experimental design is depicted in Table 1.

Table 1. The first set of mice (CPP experiment) was composed of a total of 96 animals that underwent an RSD/EXP protocol and whose anxiety-like behavior was assessed three weeks later using the EPM. Subsequently, the animals underwent CPP induced by 1 mg/kg (n = 45) or 10 mg/kg cocaine (n = 51). Finally, 10 days after extinction of the CPP induced by 1 mg/kg of cocaine, the locomotor sensitization protocol was performed.

The second set of mice (SA experiment) was composed of a total of 92 animals that underwent an RSD/EXP protocol. After 12–16 days, the animals underwent surgery to implant a catheter for the cocaine SA procedure. After three days of post-surgery recovery the SA protocol took place.

The third set of mice (Brain samples) was composed of 20 animals that underwent an RSD/EXP protocol and were sacrificed for brain sampling three weeks later.

### 2.4. Apparatus and procedures

#### 2.4.1. Procedure of social defeat

Each episode of SD consisted of three phases, which began by introducing the "intruder" (the experimental animal) into the home cage of the "resident" (the aggressive opponent) for 10 min (Tornatzky and Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interaction and species-typical threats from the male aggressive resident, thus leading to instigation and provocation (Covington and Miczek, 2001). The wire mesh was then removed from the cage to allow confrontation between the two animals for a 5-min period. 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 SD. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). All agonistic encounters were videotaped to confirm social defeat of the intruder mice and to perform an ethological analysis of the attack behaviors (duration) of the resident mice. The exploration groups followed the same protocol, but without the presence of "resident" mice: the mouse was placed in a new cage enclosed with a wire mesh for 10 min, after which the mesh was removed for 5 min and then returned again for the 10 last minutes of each exploration session.

#### 2.4.2. Elevated plus maze-EPM

The elevated plus maze (EPM) test was carried out essentially following the procedure described by Daza-Losada and cols. (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 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. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The measurements recorded during the test period were number of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. The time and percentage of time spent in the open arms and the number of open arm entries are generally used to characterize the anxiolytic effects of drugs. In addition, the number of closed and total entries indicates motor activity.

#### 2.4.3. Conditioned place preference-CPP

Place conditioning consisted of three phases and took place during the dark cycle (Maldonado et al., 2006), following an unbiased procedure in initial spontaneous preference terms (for a detailed explanation of the procedure (see Daza-Losada et al., 2009). For place conditioning, twelve identical Plexiglas boxes with a black and white equally sized compartments (30.7 cm × 31.5 cm × 34.5 cm) separated by a grey

**Table 1**
Experimental design.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1st set of mice CPP** | | **Social defeat / Exploration** | | | | | | **CPP (1 or 10 mg/kg cocaine)** | | | Locomotor sensitization |
| | | **1st** | **2nd** | **3rd** | **4th** | **3 weeks** | **EPM** | **Pre-C test** | **Conditioning** | **Post-C test** | **Induction Test** |
| | **PND** | 47 | 50 | 53 | 56 | | 76 | 77-78-79 | 80-83 | 84 | > 94 |
| **2nd set of mice SA** | | **Social defeat / Exploration** | | | | | **Catheter implantation** | **Cocaine self-administration** | | | |
| | | **1st** | **2nd** | **3rd** | **4th** | | | **Acquisition phase** | **Extinction phase** | **Reinstatement** | |
| | **PND** | 47 | 50 | 53 | 56 | | 68-72 | 77-88 | 89-114 | 117 | |
| **3rd set of mice Brain samples** | | **Social defeat / Exploration** | | | | | | | | | |
| | | **1st** | **2nd** | **3rd** | **4th** | | **Brain Samples** | | | | |
| | **PND** | 47 | 50 | 53 | 56 | | 77 | | | | |

central area (13.8 cm × 31.5 cm × 34.5 cm) and containing photoelectric cells were used (Cibertec, S.A., Madrid, Spain). In brief, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-min period was recorded. Mice showing a strong unconditioned aversion (less than 33% of the time spent in either compartments) or preference (more than 67%) for any compartment were excluded from the study.

In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 min. After a 4-h interval, they received cocaine immediately before being confined to the drug-paired compartment for 30 min. In the third phase or post-conditioning (Post-C), the time spent by the untreated mice in each compartment during a 15-min observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates induction of an aversion.

All groups in which a preference for the drug-paired compartment was established underwent an extinction session every 72 h, which consisted of placing mice in the apparatus for 15 min. This was repeated until the time spent in the drug-paired compartment by each group was similar to that of Pre-C.

The effects of non-contingent administration of a priming dose of cocaine were evaluated 24 h after confirmation of extinction. Reinstatement tests were the same as for Post-C (free ambulation for 15 min), except that mice were tested 15 min after administration of the drug (half of the dose used for conditioning). After this first reinstatement test, the groups that demonstrated reinstatement—i.e. a positive significant difference between the time spent in the drug-paired compartment in the reinstatement and last extinction tests—were retested until a new extinction was confirmed. The following day, the effects of the priming (a quarter of the dose used for conditioning) on reinstatement of place preference were evaluated following the procedure described previously. This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

#### 2.4.4. Locomotor sensitization testing

Locomotor sensitization induced by cocaine was measured by evaluating the movements of mice inside the cage by means of photocell beam breaks. Ten days after extinction of the CPP in the group of animals conditioned with 1 mg/kg cocaine, mice were assigned to a cocaine or saline condition and injected with a dose of 25 mg/kg per day of either on three consecutive days in their home cages. After five days with no treatment all mice were injected with 10 mg/kg cocaine and placed for the first time in the actimeter. Locomotor activity was automatically measured by an actimeter (Cibertec S.A., Madrid, Spain) consisting of eight cages (33 × 15 × 13 cm), each with eight infrared lights located in a frame around the cage. In this apparatus, beams are positioned on the horizontal axis 2 cm apart, at a height just above the bottom of the cage (body level of mice). The different frames are placed 4 cm apart and, since they are opaque, prevent animals from seeing each other while allowing them to hear and smell conspecifics being tested at the same time.

#### 2.4.5. Operant cocaine self-administration

The SA experiments were carried out in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Cibertec S.A., Madrid, Spain) containing two holes; one was defined as active and the other as inactive. Nose-poking in the active hole produced a cocaine infusion that was paired with two light stimuli, one placed inside the nose-poke and the other above the active hole. Nose-poking in the inactive hole had no consequences. The side on which active/inactive holes were placed were counterbalanced. The chambers were placed in sound- and light-attenuated boxes retrofitted with fans to provide ventilation and white noise.

Surgery for catheter implantation. The surgery to implant the intravenous catheter was performed as described previously (Soria et al., 2008; López-Arnau et al., 2017). Surgery was carried out in 24 animals in the exploration/saline group (EXP-SAL), 25 animals in the repeated social defeat/saline group (RSD-SAL), 19 animals in the exploration/oxytocin group (EXP-OXT), and 24 in the repeated social defeat/oxytocin group (RSD-OXT). In short, mice were anaesthetized with a mixture of ketamine/xylazine (50 mg/ml, 10 mg/ml, administrated in a volume of 0.15 ml/10 g) and then implanted with a jugular catheter. Animals were treated with analgesic (meloxicam 0.5 mg/kg; i.p., administrated in a volume of 0.10 ml/10 g body weight) and antibiotic solution (Enrofloxacin 7.5 mg/kg; i.p., administrated in a volume of 0.03 ml/10 g body weight). After surgery, animals were housed individually, placed over electric blankets, and allowed to recover.

*2.4.5.1. Acquisition.* At least 3 days after surgery, animals were trained (on a fixed ratio 1, FR1) to self-administer cocaine (0.5 mg/kg per infusion) during 10 daily sessions (2 h). Cocaine infusion of 20 µl was delivered over 2 s via a syringe collocated on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA), connected via Tygon tubing (0.96 mm outer diameter, Portex Fine Bore Polythene Tubing, Portex Limited, Kent, England) to a liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and the intravenous catheter. In order to avoid overdosing, mice received a maximum of 150 infusions, and each infusion was followed by a 15-s time-out period in which no cocaine infusions were delivered. At the beginning of each session, the house light was ON for 3 s and OFF during the rest of the experiment. The session started with a priming injection of cocaine and a 4-s light cue situated above the active hole. The number of infusions (responses during time in) in the active and the inactive hole were counted. The criteria for the acquisition of a stable SA were: 5 or more responses in the active hole; more than 65% of responses in the active hole; and a stable response with less than 30% deviation from the mean of the total number of cocaine infusions obtained on two consecutive days (70% of stability).

*2.4.5.2. Extinction.* Only animals that fulfilled the acquisition criteria underwent the extinction phase. In this phase, the experimental conditions were the same as those in the acquisition phase, except that the active hole produced no consequences and the stimulus light was turned off. The number of extinction sessions varied depending on each animal. The number of responses (during time in) in the active and the inactive holes were counted. The extinction criteria were: less than 30% of active responses on the day of maximum consumption during the acquisition phase, and a stable response with less than 30% deviation from the mean of the total number of responses obtained on two consecutive days (70% of stability).

The patency of the intravenous catheters was evaluated at the end of the first extinction session by infusion of 0.1 ml of tiobarbital (thiopental sodium; 5 mg/ml; i.v.; B. Braun Medical, S.A. Rubí, Barcelona, Spain). If signs of anesthesia did not appear within the first 3 s, the mouse was removed from the experiment.

*2.4.5.3. Reinstatement.* Only animals who fulfilled the extinction criteria were subjected to the reinstatement phase. The day after mouse met said criteria, reinstatement was induced by a priming injection of cocaine (10 mg/kg; i.p.). Immediately following cocaine administration, the mouse was placed in the self-administration box to initiate the reinstatement procedure. The number of responses (during time in) in the active- and the inactive hole were counted. The reinstatement session was identical to the acquisition sessions, only that animals did not receive the drug infusions.

*2.4.6. Sample collection and BDNF quantification*

Five animals per group - exploration/saline (EXP-SAL), repeated social defeat/saline (RSD-SAL), exploration/oxytocin (EXP-OXT) and repeated social defeat/oxytocin (RSD-OXT) groups - were sacrificed by cervical dislocation. The brains were immediately removed from the skull and placed on a cold plaque. Cerebellum and olfactory bulbs were eliminated, and the different brain areas of interest were dissected (medial PFC, STR and HIP). Brain tissues samples were stored immediately at −80 °C until the BDNF assay was performed. Samples were homogenized in cold lysis buffer (NaCl 0.15 M, EDTA 0.001 M, Tris pH 7.4 0.05 M, TX-100 1%, Glycerol 10%) supplemented with protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). For BDNF quantification, the sandwich-style ELISA using the Emax ImmunoAssay System (Promega, Madrid, Spain) was performed according to the manufacturer's instructions. The protein concentration of each sample was evaluated using the DC Protein Assay (Biorad, Madrid, Spain) to determine the number of picograms of BDNF per 100 mg of protein.

*2.5. Statistical analyses*

For the CPP induced by 1 mg/kg of cocaine, the time spent in the drug-paired compartment was analyzed using an ANOVA with a between-subjects variable— Treatment, with four levels (SAL-EXP, SAL-RSD, OXT-EXP and OXT-RSD)—and a within-subjects variable—Days, with two levels (Pre-C and Post-C). For the 10 mg/kg cocaine CPP, the data was analyzed with a three-way ANOVA with two between-subjects variables— Treatment, with two levels (SAL and OXT) — and Stress, with two levels (RSD and EXP) and a within-subjects variable—Days, with two levels (Pre-C and Post-C). In the groups showing CPP, extinction and reinstatement values were analyzed by a Student's t-test. The time required for the preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test with Breslow (generalized Wilcoxon) comparisons when appropriate (Daza-Losada et al., 2009). Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was considered to be extinguished when a mouse spent 380 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 370 s). When the preference was not extinguished in an animal, it was assigned the number of days required for extinction for the group as a whole.

Data from the locomotor sensitization test were analyzed using a two-way ANOVA with two between-subjects variables – Treatment with four levels (SAL-EXP, SAL-RSD, OXT-EXP and OXT-RSD) - and Induction-treatment, with two levels (Saline and Cocaine). For the analysis of the EPM data a one-way ANOVA with a between-subjects variable— Treatment, with four levels (SAL-EXP, SAL-RSD, OXT-EXP and OXT-RSD) was performed. For the analysis of the data of intruder and resident behavior during social defeat a one-way ANOVA with a between-subjects variable— Treatment, with two levels (SAL-RSD and OXT-RSD) - was performed. In all cases, subsequent post-hoc comparisons were performed using the Bonferroni test. Data from the BDNF levels were analyzed using a two-way ANOVA with Treatment and Stress as independent factors, and the concentration of BDNF as the dependent variable.

Data from the acquisition of cocaine SA were analyzed using a four-way ANOVA with Treatment (SAL and OXT) and Stress (RSD and EXP) as between-subjects variables and Hole (Active or Inactive) and Days as within-subjects variables. For the extinction and reinstatement phases, we compared the responses obtained on the first extinction day, last extinction day and day of reinstatement. For this comparison, a four-way ANOVA was calculated using the same variables. For the result of total cocaine intake during acquisition, the day of acquisition and the day of extinction, two-way ANOVAs were calculated using the variables Treatment and Stress. For the analysis of the percentages of acquisition, extinction and reinstatement respectively, chi-squared tests were calculated. Subsequent post-hoc analyses were performed when they corresponded using the Bonferroni test.

All statistical analyses were performed using SPSS Statistics v23. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

## 3. Results

*3.1. Oxytocin does not decrease the behavioral response of resident mice during social defeat*

The ANOVA performed for the intruder mice's behavior data during the first episode of social defeat did not reveal any significant effects. In contrast, the analysis of the fourth social defeat revealed an effect of the variable Treatment [$F(2,13) = 4.626$; $p = 0.03$], and the post-hoc analysis showed that oxytocin-treated mice spent less time in avoidance behavior ($p = 0.007$) than animals injected with saline.

With respect to the behavior of resident mice, the ANOVA of the first defeat data did not reveal any significant effect of the variable Treatment, while the analysis of the fourth social defeat did show such an effect [$F(2,13) = 14.525$; $p < 0.001$]. Post-hoc analysis revealed that resident mice confronted with animals pretreated with oxytocin spent more time engaged in threat behavior ($p < 0.001$) but had a tendency to spend less time in attack ($p = 0.061$) (See Table 2).

Table 2. Oxytocin does not decrease the behavioral response of resident mice during social defeat (n = 8). Data are presented as mean of time in seconds (s). ± S.E.M. Bonferroni test **p < 0.01; ***p < 0.001 significant difference with respect to the corresponding saline group.

*3.2. Oxytocin prevents the long-term anxiogenic effects of repeated social defeat*

The data of the EPM test are presented in Table 3. The ANOVA of the time spent in the open arms [$F(3,52) = 8.126$; $p < 0.01$]; percentage of time spent in the open arms [$F(3,52) = 6.553$; $p < 0.001$]; percentage of entries into the open arms [$(3,52) = 2.782$; $p = 0.05$]; number of entries into the closed arms [$(3,52) = 9.158$; $p < 0.01$] revealed a significant effect of the variable Treatment. Post-hoc analyses showed that socially defeated animals pretreated with saline spent less time and a lower percentage of time in the open arms than animals in any of the exploration groups ($p < 0.001$), and socially defeated animals pretreated with OXT ($p < 0.05$). A lower percentage of entries into the open arms was registered in socially defeated animals pretreated with saline compared with defeated animals pretreated with oxytocin ($p < 0.05$). On the other hand, socially defeated animals under saline treatment performed a higher number of entries into the closed arms than the rest of the groups ($p < 0.001$).

Table 3. Administration of oxytocin before each social defeat prevents the long-lasting anxiogenic effect in the EPM (SAL-EXP n = 15; SAL-RSD n = 15; OXT-EXP n = 14; OXT-RSD n = 12). Data are

**Table 2**
Behavior of resident and intruder mice during 5-min agonistic encounters.

| Social Defeat | Saline treated | | Oxytocin treated | |
|---|---|---|---|---|
| | 1st | 4st | 1st | 4th |
| | *Intruder mice* | | | |
| Avoidance (s) | 16 ± 3 | 25 ± 3 | 15 ± 5 | 10 ± 2 ** |
| Defense/Submission (s) | 58 ± 10 | 74 ± 12 | 68 ± 8 | 51 ± 14 |
| | *Resident mice* | | | |
| Threat (s) | 9 ± 3 | 5 ± 2 | 9 ± 3 | 16 ± 2 *** |
| Attack (s) | 29 ± 6 | 28 ± 5 | 26 ± 7 | 15 ± 4 |

**Table 3**
Long term effects of RSD on anxiety like behavior.

| | SAL-EXP | SAL-RSD | OXT-EXP | OXT-RSD |
|---|---|---|---|---|
| Time in OA | 89 ± 8 | 42 ± 5***+ | 61 ± 2 | 68 ± 9 |
| %Time OA | 34 ± 3 | 17 ± 2***+ | 33 ± 1 | 28 ± 4 |
| %Entries OA | 35 ± 3 | 25 ± 5+ | 37 ± 1 | 42 ± 4 |
| Closed entries | 37 ± 7 | 81 ± 13***+++ | 31 ± 2 | 32 ± 4 |

presented as mean values of time in seconds (s). ± S.E.M. Bonferroni test ***$p < 0.001$ significant difference from non-stressed control groups (SAL-EXP and OXT-EXP); + $p < 0.05$, +++ $p < 0.0001$ significant difference with respect to RSD animals treated with OXT (OXT-RSD).

### 3.3. Oxytocin modifies the expression of BDNF in the prefrontal cortex, striatum and hippocampus of treated mice

A two-way ANOVA of BDNF levels in the PFC revealed an effect of the variable Treatment [$F(1,20) = 9.006$; $p < 0.01$] and the interaction between Treatment and Stress [$F(1,20) = 8.063$; $p < 0.05$] (see Fig. 1A). A Bonferroni post-hoc test revealed a lower expression of BDNF in RSD-OXT vs. RSD-SAL mice ($p < 0.01$). In STR, a two-way ANOVA showed an effect of Treatment [$F(1,20) = 13.647$; $p < 0.01$] and Stress [$F(1,20) = 8.639$; $p < 0.01$], but no significant interaction between the two factors (see Fig. 1B). The effect of Treatment observed demonstrates that animals who received OXT expressed higher BDNF levels than those treated with saline in this brain area. The Stress effect reveals higher levels of BDNF in the STR in animals that experienced RSD vs. EXP. In the HIP, two-way ANOVA of BDNF levels indicated an effect of Treatment [$F(1,21) = 20.647$; $p < 0.001$], Stress [$F(1,21) = 8.281$; $p < 0.01$] and the interaction between these factors [$F(1,21) = 5.702$; $p < 0.05$] (see Fig. 1C). The post-hoc analysis shows that the levels of BDNF were higher in the RSD-OXT group compared with the EXP-SAL ($p < 0.001$); RSD-SAL ($p < 0.001$) and EXP-OXT groups ($p < 0.01$), respectively.

### 3.4. Oxytocin blocks the increase in the conditioned rewarding effects of a subthreshold dose of cocaine (1 mg/kg) induced by social defeat stress

The ANOVA of the CPP data (see Fig. 2) showed a significant effect of the interaction Days and Treatment [$F(3,50) = 3.930$; $p = 0.014$]. As expected, socially defeated animals pretreated with saline (SAL-RSD) developed CPP, since they spent more time in the drug-paired compartment in the Post-C than in the Pre-C test ($p < 0.001$). This place preference was not developed in defeated mice pre-treated with oxytocin (OXT-RSD).

### 3.5. Oxytocin decreases the number of sessions required to extinguish the place preference induced by 10 mg/kg cocaine in both stressed and non-stressed animals

The ANOVA of the CPP induced by 10 mg/kg of cocaine (see Fig. 3) showed a significant effect of the variable Days [$F(1,47) = 50.898$; $p < 0.001$]. Post-hoc comparisons showed that all groups developed a significant preference for the drug-paired compartment after being conditioned with 10 mg/kg of cocaine.

All groups were subjected to CPP extinction sessions, and the Kaplan–Meier analysis revealed that more time was required to achieve extinction in the SAL-RSD (17 sessions) than in those treated with oxytocin OXT-RSD ($\chi 2 = 6.667$; $p = 0.01$) (9 sessions) or OXT-EXP ($\chi 2 = 6.418$; $p = 0.011$) (9 sessions) (see Fig. 4). Reinstatement of the preference after a priming dose of 5 mg/kg of cocaine was achieved in both saline groups - SAL-EXP and SAL-RSD ($p < 0.05$) - and in socially

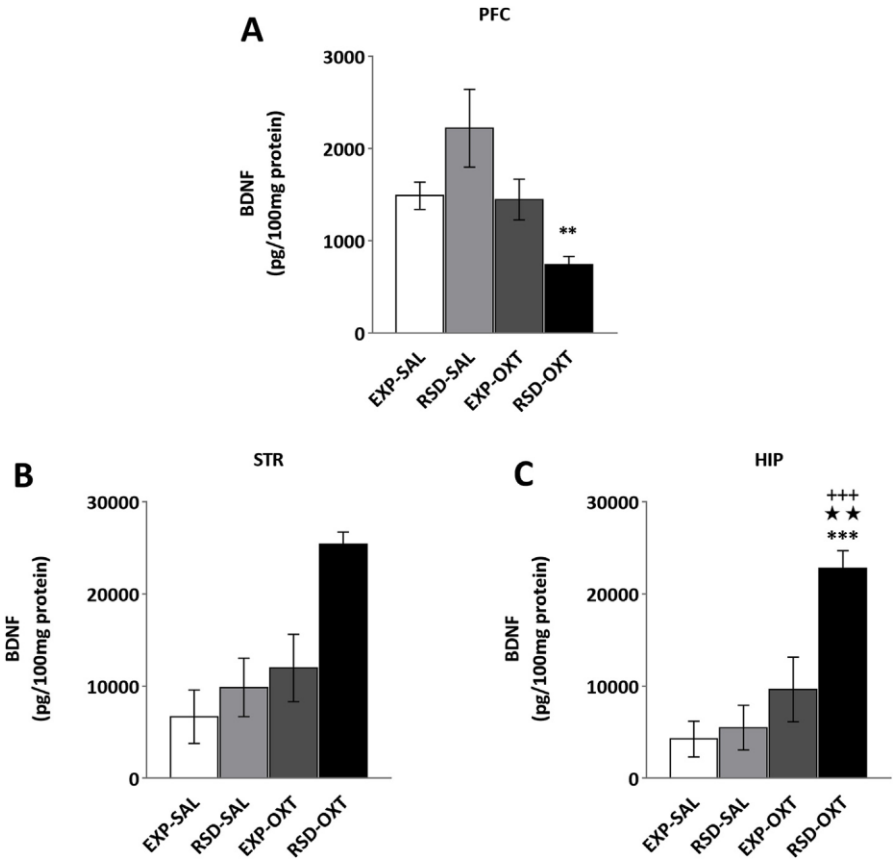


**Fig. 1. Effects of RSD and/or OXT treatment on BDNF levels.** Mean of BDNF levels (pg/100 mg protein) in (A) PFC, (B) STR and (C) HIP. Two-way ANOVA and subsequent Bonferroni post-hoc test. ★★$p < 0.01$, versus EXP-OXT group. **$p < 0.01$, ***$p < 0.001$, versus RSD-SAL group. +$p < 0.001$ versus EXP-SAL group. Data are expressed as mean of BDNF (pg/100 mg protein) ± S.E.M. ($n = 5$).

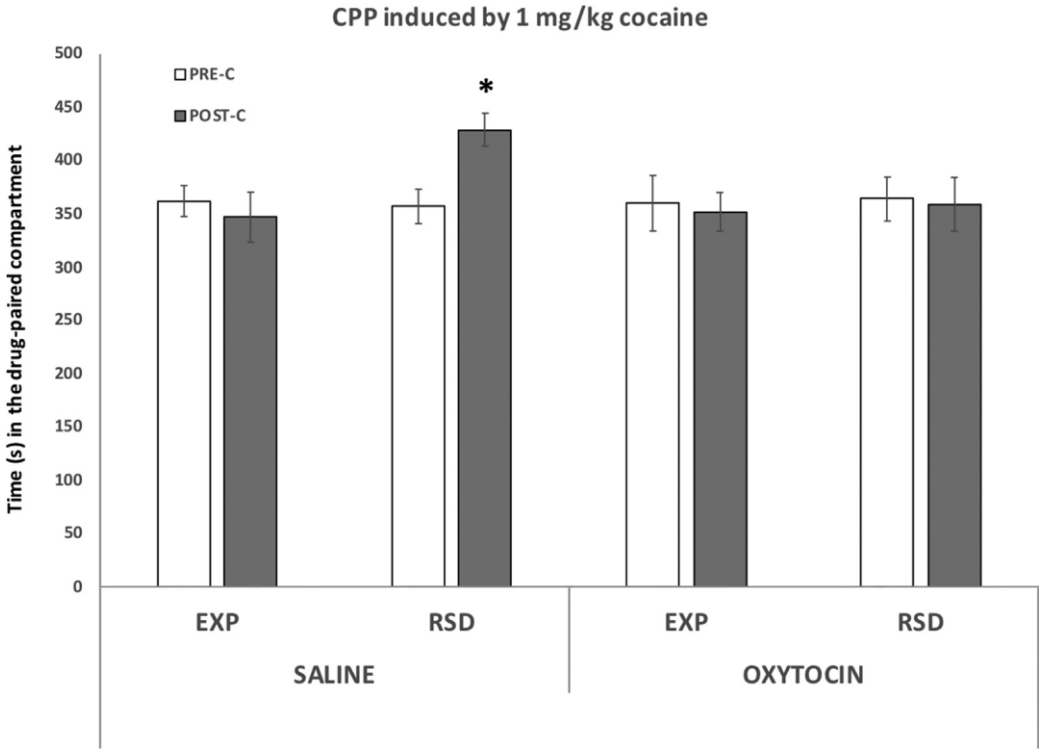


**Fig. 2. Administration of oxytocin before each social defeat episode blocked acquisition of the CPP induced by 1 mg/kg of cocaine in defeated mice**. Before the social stress protocol animals were randomly assigned to the following groups according to treatment: saline (SAL-EXP n = 14; SAL-RSD n = 15); Oxytocin 1mg/kg (OXT-EXP n = 12; OXT-RSD n = 13). The bars represent the time (s) spent in the drug-paired compartment before conditioning sessions in the PRE-C test (white bars), and after conditioning sessions in the POST-C test (dark grey bars). Data are presented as mean values ± S.E.M. Bonferroni`s test *p < 0.05 significant difference in the time spent in the drug-paired compartment versus PRE-C.

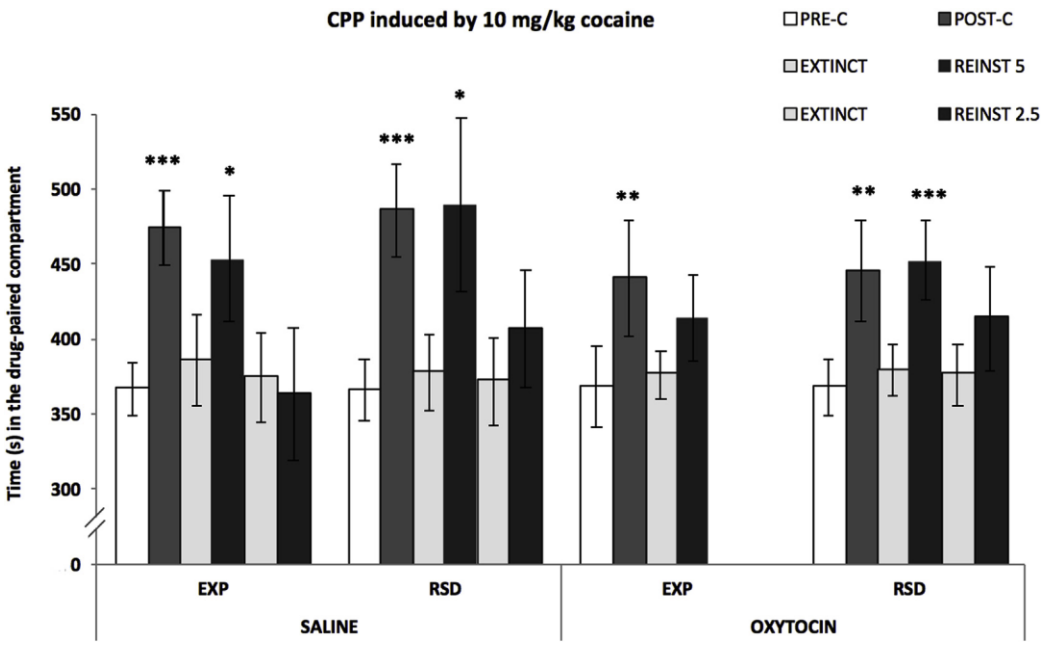


**Fig. 3. Administration of oxytocin before each social defeat does not affect acquisition of the CPP induced by 10 mg/kg of cocaine**. Before the social stress protocol animals were randomly assigned to one of the following groups according to the pre-treatment they received: saline (SAL-EXP n = 14; SAL-RSD n = 12); or Oxytocin 1 mg/kg (OXT-EXP n = 12; OXT-RSD n = 13). The bars represent the time (s) spent in the drug-paired compartment before conditioning sessions in the PRE-C test (white bars), and after conditioning sessions in the POST-C test (dark grey bars). Data presented as mean values ± S.E.M. Bonferroni's test *p < 0.05; **p < 0.01; ***p < 0.001 significant difference in the time spent in the drug-paired compartment versus PRE-C.

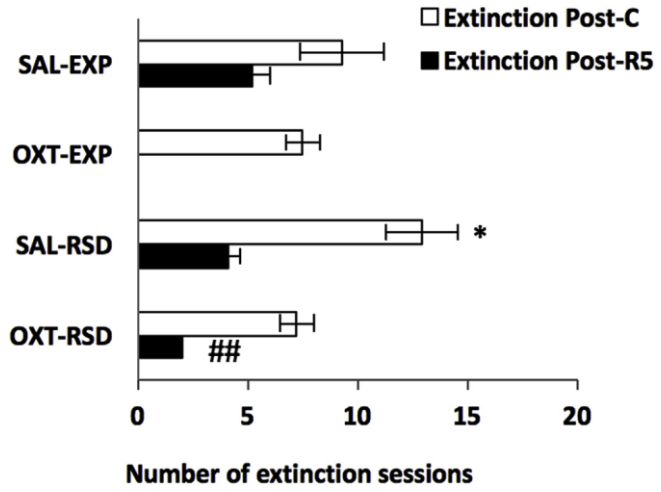


**Fig. 4. Effect of oxytocin administration before each social defeat on the number of sessions needed to extinguish the preference induced by 10 mg/kg of cocaine.** White bars represent the preference induced by 10 mg/kg cocaine while grey bars represent the preference reinstated with a priming dose of 5 mg/kg cocaine. *p < 0.05 significant difference in the number of extinction sessions versus the oxytocin (OXT) groups. Bonferroni's test ###p < 0.001 significant difference in the number of extinctions sessions versus saline (SAL) groups.

defeated animals pretreated with oxytocin OXT-RSD ($p < 0.001$). No reinstatement was observed in non-stressed mice treated with oxytocin (SAL-EXP). Again, differences were observed in the time needed to extinguish this preference, since the OXT-RSD group required less extinction sessions (2 sessions) than the SAL-EXP ($\chi 2 = 10.193$; $p = 0.001$) (8 sessions) and SAL-RSD ($\chi 2 = 10.111$; $p = 0.001$) (6 sessions) groups.

### 3.6. Oxytocin reduces the increased cocaine locomotor sensitization induced by social defeat stress

Fig. 5 displays the scores during the first 10 min of induced motor activity in animals after a motor sensitization protocol. The ANOVA revealed an effect of the variables Treatment [$F(3,50) = 4.111$; $p < 0.01$] and Induction-treatment [$F(1,50) = 38.854$; $p < 0.001$]. Post-hoc analyses showed that animals treated with cocaine during the induction phase (C-SAL-EXP, C-SAL-RSD, C-OXT-EXP, and C-OXT-RSD) were more reactive to a challenge dose of 5 mg/kg than those treated with saline (S-SAL-EXP, S-SAL-RSD, S-OXT-EXP, and S-OXT-RSD) ($p < 0.001$). Additionally, RSD induced cross-sensitization to cocaine, since saline defeated animals (S-SAL-RSD) exhibited higher levels of motor activity than their non-stressed counterparts (S-SAL-EXP) ($p < 0.05$), an effect that was not registered in defeated animals pretreated with oxytocin (see Fig. 5).

### 3.7. Repeated social defeat increases cocaine intake in the acquisition phase of cocaine self-administration

Following the criteria of acquisition, the percentage of mice that acquired cocaine self-administration behavior was 47.8% for EXP-SAL ($n = 23$), 47.6% for RSD-SAL ($n = 21$), 41.2% for EXP-OXT ($n = 17$) and 45% for RSD-OXT ($n = 20$). Chi-squared analysis showed no significant differences between the groups ($\chi 2 = 0.217$; $p > 0.05$). A two-way ANOVA of total cocaine intake (see Fig. 6A) showed a main effect of Stress [$F(1,33) = 4.604$; $p < 0.05$], indicating that mice exposed to RSD consumed more cocaine than non-stressed mice. In relation to the day of acquisition (see Fig. 6B), the two-way ANOVA showed no significant differences between groups. The cocaine infusions received throughout the acquisition phase (Fig. 6C) were calculated by means of a four-way ANOVA (Treatment, Stress, Days and Holes as factors of variations), revealing a main effect of Days [$F(9,297) = 3.538$; $p < 0.001$], Hole [$F(1,33) = 32.630$; $p < 0.001$], a tendency for Stress factor [$F(1,33) = 3.725$; $p = 0.062$]; interaction between Hole and Stress [$F(1,33) = 5.428$; $p < 0.05$] and Hole and Days [$F(9,297) = 4.941$; $p < 0.001$]; and a tendency between Days and Stress [$F(9,297) = 1.893$; $p = 0.053$]. Finally, we obtained an interaction between Days, Hole and Stress [$F(9,297) = 2.263$, $p < 0.05$]. Bonferroni post-hoc analysis revealed that animals exposed to RSD engaged in a higher number of active infusions than non-stressed mice ($p < 0.05$). Bonferroni's post-hoc analysis of Hole and Days showed that RSD mice were capable of discriminating between the holes from the first day of acquisition until day 10, ($p < 0.05$, in all the cases), whereas non-stressed mice discriminated only on days 3, 4, 5, 6, 8 and 9 ($p < 0.05$, in all the cases). Additionally, the results showed that mice exposed to RSD performed more active infusions than non-

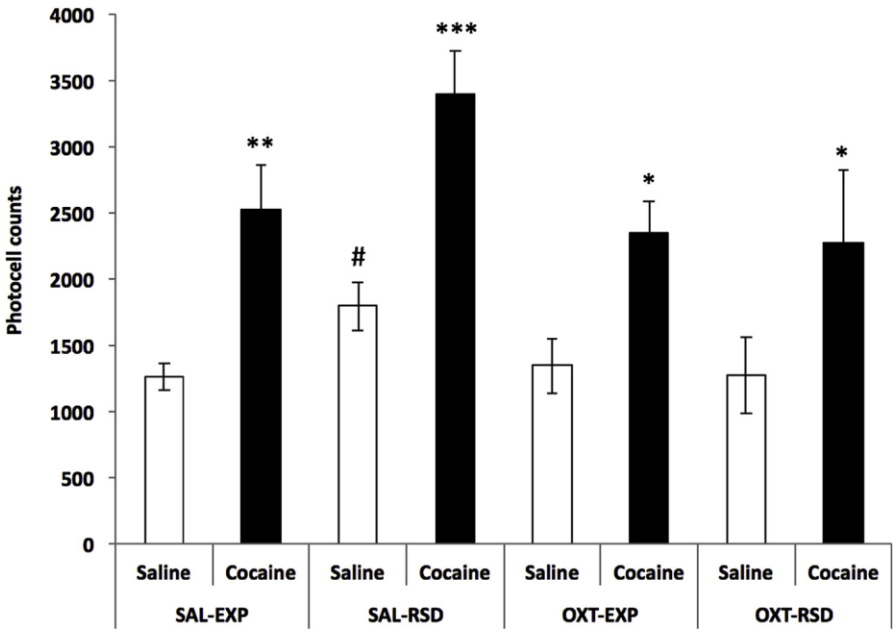


**Fig. 5. Administration of 1 mg/kg of oxytocin before each social defeat blocks the increased locomotor sensitization to cocaine induced by social defeat stress.** During the induction phase, half of the animals in each group received pretreatment with saline (S) (S-SAL-EXP n = 8; S-SAL-RSD n = 8; S-OXT-EXP n = 6; S-OXT-RSD n = 6) and the other half received 25 mg/kg cocaine per day (C) (C-SAL-EXP n = 9; C-SAL-RSD n = 8; C-OXT-EXP n = 7; C-OXT-RSD n = 6) on three consecutive days. Data presented as mean values ± S.E.M. during the first 10-min period of locomotor activity in photocell counts. Bonferroni's test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significant difference with the corresponding saline induction (S) group; # $p < 0.001$ significant difference with respect to the corresponding Exploration (EXP) group.

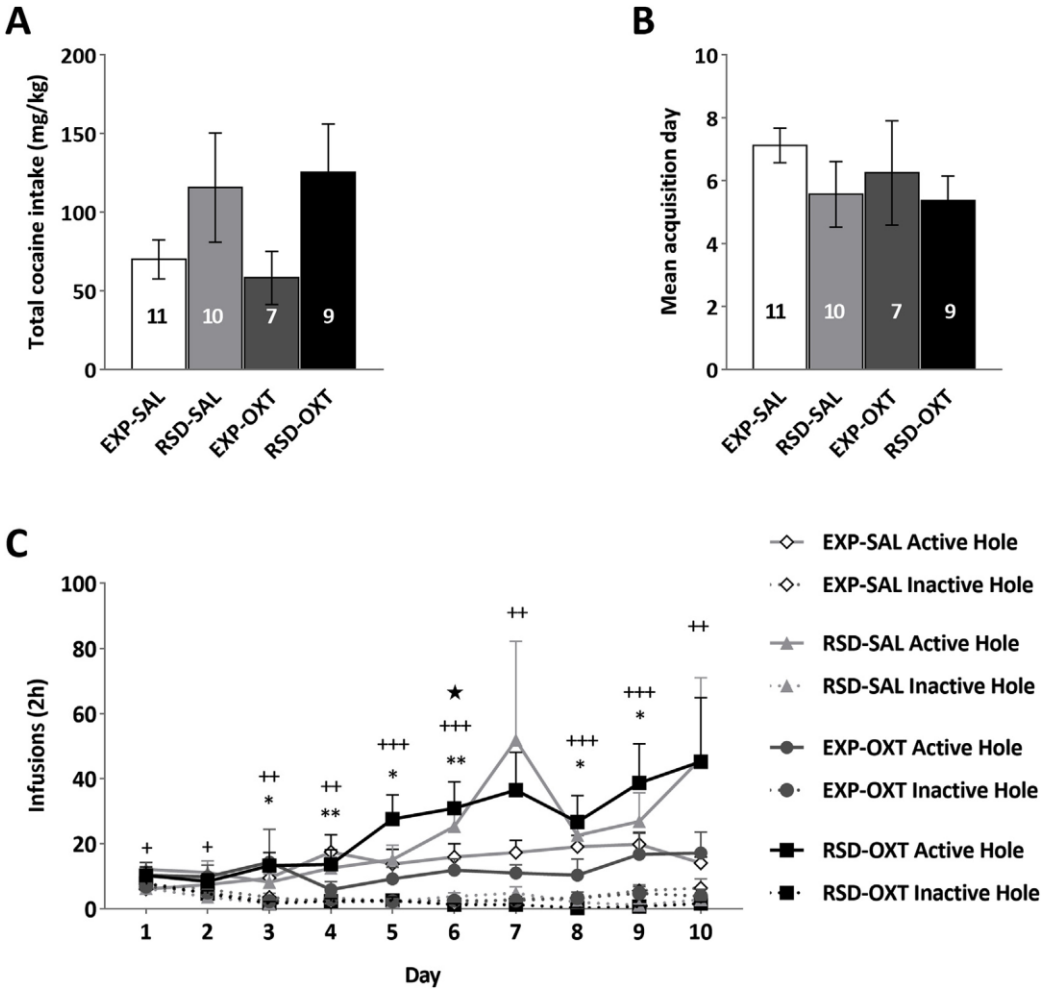


**Fig. 6. Effects of RSD and/or OXT treatment on acquisition of cocaine self-administration behavior**. (A) Mean of total cocaine intake (mg cocaine/kg mice) throughout the 10 days of the acquisition phase (2 h per session). (B) Mean of days needed to acquire self-administration behavior. (C) Mean number of infusions in the active/inactive hole during the 10-day acquisition phase (2 h per session). Four-way ANOVA and Bonferroni's post-hoc test. ★$p < 0.05$, Stress effect on active responses. +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$, discrimination between active/inactive holes among RSD mice. *$p < 0.05$, **$p < 0.01$, discrimination between active/inactive holes among EXP mice. Data are expressed as mean ± S.EM. (n = 7 to 11).

stressed mice on day 6 ($p < 0.05$). No effect of OXT treatment was observed.

### *3.8. Oxytocin administration reduces the number of days needed to extinguish cocaine seeking-behavior and reverses the cocaine-seeking induced by repeated social defeat after a cocaine priming*

The percentage of mice in which self-administration behavior was extinguished did not differ between groups ($\chi 2 = 2.091$; $p > 0.05$): 72.7% (EXP-SAL, n = 11), 70% (RSD-SAL, n = 10), 57.1% (EXP-OXT, n = 7) and 88.9% (RSD-OXT, n = 9). A two-way ANOVA of day of extinction (see Fig. 7A) demonstrated an effect of Treatment [$F(1,23) = 15.468$; $p < 0.01$], a tendency for Stress [$F(1,23) = 3.942$; $p = 0.059$] and an interaction between Treatment and Stress [$F(1,23) = 6.853$; $p < 0.05$]. Bonferroni's post-hoc test revealed that mice in the EXP-OXT group needed fewer days for cocaine-seeking behavior to be extinguished than the EXP-SAL ($p < 0.001$) and RSD-OXT groups ($p < 0.01$). To compare behaviors during the First Extinction Day, the Last Extinction Day and the Reinstatement Day (see Fig. 7B), a four-way ANOVA was performed and showed an effect of Days [$F(2,46) = 9.906$, $p < 0.001$] and Hole [$F(1,23) = 27.139$; $p < 0.001$], and interactions between Days and Stress [$F(2,46) = 3.194$; $p < 0.05$], Hole and Days [$F(2,46) = 10.938$; $p < 0.001$], Days, Treatment and Stress [$F(2,46) = 4.541$; $p < 0.05$], Hole, Treatment and Stress [$F(1,23) = 6.861$; $p < 0.05$], Days, Hole and Stress [$F(2,46) = 3.565$; $p < 0.05$] and between all the factors (Days, Hole, Treatment and Stress) [$F(2,46) = 3.390$; $p < 0.05$]. The Days effect revealed a lower number of responses during the Last Extinction Day in comparison with the First Extinction Day ($p < 0.001$) and an increased number of responses on the Reinstatement Day in comparison with the Last Extinction Day ($p < 0.01$). The Hole effect showed active responses were stronger than inactive responses ($p < 0.001$). Bonferroni's post-hoc analysis of Stress and Days demonstrated that both groups exhibited more responses during the First Extinction Day than the Last Extinction Day ($p < 0.01$ in both cases). The interaction Days and Hole showed that animals displayed more active responses during the First Extinction Day than on the Last Extinction Day ($p < 0.01$) and more active responses on the Reinstatement Day versus the Last Extinction Day ($p < 0.01$). This interaction also highlighted a discrimination between holes on all the days evaluated (First Extinction Day $p < 0.001$, Last Extinction Day $p < 0.05$ and Reinstatement Day $p < 0.001$). Interaction of Treatment, Stress and Days revealed a higher response in the RSD-OXT group in comparison with the RSD-SAL group during the Last Extinction Day ($p < 0.05$) and more responses in the RSD-SAL group than in the EXP-SAL group during the Reinstatement Day ($p < 0.01$). Moreover, EXP-

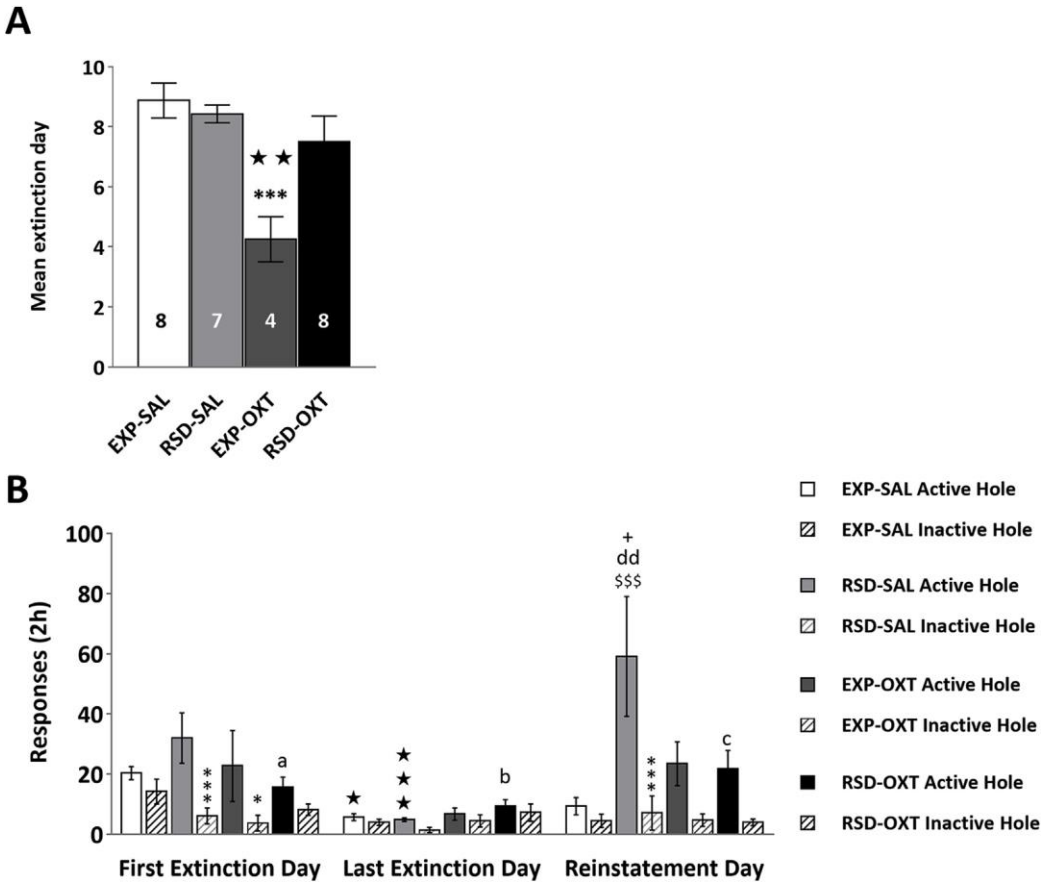


**Fig. 7. Effects of RSD and/or OXT treatment on the extinction and reinstatement of cocaine self-administration behavior**. (A) Mean of days needed to extinguish cocaine seeking-behavior. Two-way ANOVA and Bonferroni's post hoc test. ***$p < 0.001$, effect of Treatment in EXP mice. ★★$p < 0.01$, effect of Stress in animals treated with oxytocin. (B) Mean of responses in the active/inactive hole during the First Extinction Day, the Last Extinction Day and the Day of Reinstatement. Four-way ANOVA and Bonferroni's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ discrimination between active/inactive holes. ★$p < 0.05$, ★★★$p < 0.001$, active responses on the Last Extinction Day vs. active responses on the First Extinction Day. $$$$p < 0.001$, active responses on the Reinstatement Day vs. active responses on the Last Extinction Day. +$p < 0.05$, active responses on Reinstatement Day vs. active responses on the First Extinction Day. a ($p < 0.05$), effect of Treatment on the active responses of defeated mice during the First Extinction Day (a; $p < 0.05$), the Last extinction Day (b; $p < 0.05$) and Reinstatement Day (c; $p < 0.05$). dd ($p < 0.01$), Stress effect on the active responses of mice treated with saline during Reinstatement Day. Data are expressed as mean ± S.E.M. ($n = 4$ to 8).

SAL ($p < 0.01$) and RSD-SAL ($p < 0.001$) groups exhibited decreased responses on the Last Extinction Day compared with the first extinction day. However, only the RSD-SAL group displayed significantly increased responses on the Reinstatement Day compared with the Last Extinction Day ($p < 0.001$). Interaction between Treatment, Stress and Hole was stronger in the RSD-SAL group compared with EXP-SAL ($p < 0.01$) and RSD-OXT ($p < 0.05$) groups. This interaction also highlighted discrimination between holes by the RSD-SAL ($p < 0.001$) and EXP-OXT ($p < 0.05$) groups. There was a decrease in the interaction between Stress, Days and Hole during the Last Extinction Day in comparison with the First Day of Extinction, independently of the Stress experience. This interaction also shows that RSD mice displayed a preference for the active hole during the First Extinction Day ($p < 0.01$), Last Extinction Day ($p < 0.05$) and Reinstatement Day ($p < 0.001$), while in the EXP groups, this preference was evident only on the First Day of Extinction ($p < 0.05$). Bonferroni's post-hoc test of all the factors revealed more active responses on Reinstatement Day among RSD-SAL mice than in the EXP-SAL group ($p < 0.01$), demonstrating that RSD increased cocaine seeking-behavior during the reinstatement phase. We also observed a significant decrease of active responses in the EXP-SAL ($p < 0.05$) and RSD-SAL groups ($p < 0.001$) on the Last Extinction Day in comparison with the First Extinction Day. In addition, the interaction between all factors on Reinstatement day was more pronounced in RSD-SAL mice than in the EXP-SAL group ($p < 0.01$), suggesting that RSD increased vulnerability to the reinstatement of cocaine-seeking. Moreover, the RSD-SAL group was the only one capable of discriminating between holes during the day of reinstatement ($p < 0.001$), and the only one in which the number of active responses was significantly higher on Reinstatement Day compared with the Last Extinction Day ($p < 0.05$) and even with the First Extinction Day ($p < 0.05$). This result means that the RSD-SAL group was the only one in which cocaine-seeking behavior was reinstated after an i.p cocaine priming. Ultimately, RSD-OXT mice performed stronger responses than the RSD-SAL group during the Last Day of Extinction ($p < 0.05$). However, the RSD-OXT group showed weaker responses on the First Day of Extinction ($p < 0.05$) and on Reinstatement Day ($p < 0.05$) than their RSD-SAL counterparts, suggesting that OXT treatment decreased the vulnerability to reinstate cocaine-seeking induced by RSD.

## 4. Discussion

Our results confirm that elevated levels of OXT when experiencing social defeat stress can block the long-lasting effects of this type of stress. OXT counteracts the anxiety induced by RSD and modifies BDNF levels in all the structures studied. RSD induced increases in the

motivational effects of cocaine. Administration of OXT before each SD prevented RSD-induced increases in the conditioned rewarding effects of cocaine in the CPP, favored the extinction of cocaine-associated memories in both the CPP and SA paradigms, and decreased reinstatement of cocaine-seeking behavior in the SA.

Firstly, as the animals experienced the four SD episodes under the effect of OXT, it was necessary to confirm that the mice were actually socially defeated. The ethological analysis of the social encounter showed that resident mice threatened and attacked both OXT- and saline-treated mice to the same extent. Even more threats were observed during the last social encounter when the residents were confronted with mice treated with OXT. On the other hand, the intruder mice treated with OXT displayed less avoidance behavior during the last social defeat episode. In spite of these differences, we can conclude that OXT-treated mice experience similar SD to saline-treated counterparts, exhibiting similar behaviors to those observed in previous reports (Montagud-Romero et al., 2017).

It is well known that SD induces a clear anxiogenic effect (Albrechet-Souza et al., 2017; Macedo et al., 2018), which is completely blocked by OXT administration. Although data are controversial (for example, Grippo et al., 2012), many studies indicate that OXT exerts an anxiolytic effect in both sexes and even in aged rats (Balmus et al., 2018; Waldherr and Neumann, 2007). However, we did not observe any OXT effect on non-stressed mice. On the other hand, acute or chronic administration of OXT reduces anxiety-related behavior in stressed rodents (Ring et al., 2006; Slattery and Neumann, 2010; Windle et al., 1997). In agreement with our results, Lukas et al. (2011) showed that the loss of social preference and social avoidance in response to a single SD could be restored by intracerebroventricular infusion of OXT 20 min before the social preference test. In this sense, our results demonstrate that OXT administration before social stress can diminish the long-lasting increase in anxiety observed in defeated animals.

Numerous reports show that SD stress induces prolonged BDNF expression in the VTA and NAc, which have been associated with depressive-like symptoms (Berton et al., 2006; Fanous et al., 2010; Krishnan et al., 2007; Nikulina et al., 2012), although Miczek et al. (2011) observed an increase in tegmental BDNF in episodically defeated rats, whereas continuously subordinate rats showed suppressed BDNF levels. In the HIP, a reduction, an increase or a lack of changes after exposure to SD have been reported (Coppens et al., 2011; Duclot and Kabbaj., 2013; Lagace et al., 2010; Pizarro et al., 2004; Taylor et al., 2011; Tsankova et al., 2006). Previous studies in the PFC have described increases or decreases of BDNF immediately after social stress, without changes four weeks after stress (Fanous et al., 2010; Wang et al., 2018). In our study RSD did not affect BDNF in either of the structures evaluated. In previous work in our laboratory, we observed a down-regulation in proBDNF expression – a role in contrast to that of BDNF - in the NAc of defeated mice three weeks after the last social stress episode (Montagud-Romero et al., 2017). Interestingly, our results showed that RSD interacted with the OXT treatment, increasing the levels of BDNF in the STR and HIP, while paradoxically decreasing BDNF levels in the PFC.

Although there have been several reports of the positive effects of OXT in rats exposed to stress (Leuner et al., 2011; Cohen et al., 2010), few studies have focused on the relation between OXT, stress and BDNF. Dayi and coworkers (2015) described how OXT administration in rats exposed to restraint stress increased the expression of BDNF in the HIP, which correlated with an improvement of the cognitive deficit induced by stress. Therefore, in line with the present results, the amelioration of stress effects seems to be related to an increase of BDNF. Moreover, antidepressant administration also increased the expression of BDNF in the PFC, NAc and HIP after SD (Yang et al., 2016a,b). It is likely that synaptogenesis, including BDNF-TrkB signaling in the PFC and HIP, may underlie this sustained antidepressant effect. Indeed, current data suggest that BDNF plays a key role in the antidepressant response (Björkholm and Monteggia, 2016).

Although both are related to stress response, acute social stress has been shown to induce DNA methylation in OXTR but not in the BDNF gene (Unternaehrer et al., 2012). Emotional contagion-resistant mice - characterized by reduced sociability – display elevated concentrations of OXT but a reduced density of TrkB receptors in brain areas relevant to behavior (Laviola et al., 2017). Havranek et al. (2015) demonstrated that administration of OXT to the lateral ventricle of rats induces a significant increase of mRNA and protein levels of BDNF in the HIP. Additionally, Dayi et al. (2015) reported that administration of OXT to stressed animals increased the expression of BDNF in the HIP. In this way, the amelioration of the effects of stress appears to be related to an increase of BDNF. All of this evidence suggests that OXT exerts anxiolytic and antidepressant effects and decreases the stress response by modulating the CRF-HPA axis (Baracz et al., 2018). Our results demonstrate that OXT treatment increases BDNF levels in the STR and HIP; however, this increase is higher in defeated animals, suggesting that OXT exerts an antidepressant-like effect by modulating the stress response. In contrast with previous reports, we did not observe an increase in BDNF levels in the PFC after OXT treatment; in fact, the opposite was observed, suggesting that alternative mechanisms are involved in such an interaction.

The CPP paradigm evaluates the conditioned motivational properties of drugs (Tzschentke, 2007), and is also used to test the reinstatement of drug-seeking after extinction (Aguilar et al., 2009). In accordance with previous reports, socially defeated mice underwent a long-lasting increase in the conditioned rewarding effects of cocaine, developing CPP with a non-effective dose of cocaine (Ferrer-Pérez et al., 2018). On the other hand, all groups conditioned with an effective (10 mg/kg) dose of cocaine in the CPP showed a preference that was reinstated with successive smaller doses of cocaine, as previously reported (Maldonado et al., 2006; Montagud-Romero et al., 2016b; Rodríguez-Arias et al., 2009). OXT administration before each SD blocked the increased preference observed in defeated mice conditioned with 1 mg/kg of cocaine and induced a faster extinction of the memories associated with 10 mg/kg of cocaine CPP in stressed animals. Although no differences in reinstatement were observed in defeated mice after OXT treatment, non-stressed animals pretreated with OXT did not present reinstatement with a priming dose of 5 mg/kg, in contrast to controls, which did.

In agreement with previous reports, SD increased vulnerability to cocaine SA (e.g. Boyson et al., 2011; Burke and Miczek, 2015; Leonard et al., 2017; Yap et al., 2015). Socially defeated mice consumed more cocaine than non-stressed animals, with no OXT effect being detected. However, OXT administration reduced the number of days needed to extinguish cocaine-seeking behavior. The number of active responses fell between the first and last extinction day in all the groups, except for the OXT-treated defeated group, which showed a reduced number of responses from the first day of extinction. In response to a cocaine-priming dose, only defeated saline-treated animals showed an increased number of active responses, while OXT treatment reverted this effect. Several reports have pointed out that acute OXT administration reduces cocaine intake and reinstatement of cocaine SA (Leong et al., 2016, 2017; Sarnyai and Kovács, 1994; Zhou et al., 2014), but given that in our experiments OXT was administered weeks before initiating the procedure, we believe that OXT does not affect cocaine SA, but rather only the changes induced by RSD.

OXT prevented the increase in the conditioned rewarding effects of cocaine when a non-effective dose was administered in the CPP, but it did not modify the enhanced cocaine SA induced by RSD. It also decreased cocaine-priming-induced reinstatement of SA, but it did not affect reinstatement of the preference in the CPP. Both paradigms are used to evaluate the motivational value of a drug, but each assesses different aspects of drug-induced reward and, thus, different characteristics of relapse and addictive behavior. SA evaluates the primary rewarding properties of drugs using operant conditioning, where a

reinstatement after extinction implies the restoration of a concrete operant response. On the other hand, CPP is based on Pavlovian conditioning and it evaluates cue-elicited drug-taking behavior by assessing the incentive value of drug-associated cues and the reinstatement of CPP (consisting of the reappearance of approach behavior towards a drug-associated context) (Aguilar et al., 2009). Interestingly, in both paradigms, OXT accelerates the time needed to extinguish operant behavior or conditioned preference. Therefore, we can affirm that OXT diminishes the potency of the memories associated with drug use. Numerous reports have shown that OXT modifies learning and memory processes, mostly through hippocampal and related limbic mechanisms (Gaffori and De Wied, 1988; Gard et al., 2012; Kovács et al., 1979). However, OXT has a long-term inhibitory effect during acquisition, retention, consolidation and retrieval (Kovács and de Wied, 1994 for review; Boccia et al., 1998; Boccia and Baratti, 2000). More importantly, OXT seems to play a role in behavioral flexibility and adaptive responses (Chini et al., 2014), and may increase the cognitive flexibility necessary to acquire new learning during the extinction process.

Increased motor response after intermittent drug administration is defined as behavioral sensitization (Kalivas and Stewart, 1991; Steketee and Kalivas, 2011). As expected, all the groups that received cocaine pretreatment in our experimental protocol developed cocaine motor sensitization. In addition, SD induced cross-sensitization with cocaine. Socially defeated mice pretreated with saline showed comparable enhancement of motor activity to that observed in those pretreated with cocaine (Ferrer-Pérez et al., 2018; Kalivas et al., 1998; Lu et al., 2001). As with the effect observed in our CPP and SA experiments, OXT administration prior to each social defeat completely abolished cross-sensitization, without affecting cocaine-induced sensitization.

Our results demonstrate that OXT is capable of reverting the long-lasting increase in anxiety, in sensitivity to the motivational value of cocaine, in cross-sensitization, and in BDNF changes induced by social stress. In line with our results, several reports have indicated that OXT represents a potential new therapeutic approach for stress-induced psychiatric disorders (Ebner et al., 2000; Engelmann et al., 1999; Litvin et al., 2011; Wang et al., 2018). Also, in agreement with our results, a recent report showed that a single dose of OXT, systemically or within the bed nucleus of the stria terminalis, reverses stress-induced social avoidance in female mice (Duque-Wilckens et al., 2018). Based on previous reports (see review Sarnyai and Kovács, 2014), we propose that OXT exerts this effect independently of other mechanisms affecting the stress response, exerting an action on learning and memory processes associated with cocaine. Cocaine induces synaptic plasticity in neural systems that are vital for the development of addiction (reviewed by Hyman et al., 2006; Malenka and Bear, 2004), modifying a number of signaling cascades, including BDNF, whose elevated expression has been associated with susceptibility to drugs of abuse (Berhow et al., 1995; Flanagin et al., 2006; Guillin et al., 2001; Hall et al., 2003; Thomas et al., 2008; Tsai, 2007).

## 5. Conclusions

In conclusion, our results show that the long-lasting effects of RSD on anxiety and the motivational effects of cocaine are counteracted if OXT is administered before this type of stress is experienced. We hypothesize that, among other mechanisms, alterations in BDNF expression are involved in these protective effects. In this way, our results confirm the potential protective effects of OXT against the pathogenic effects of social stress.

## Conflicts of interest

This work has not been published previously and is not under consideration for publication elsewhere. The authors have no possible conflict of interest in the carrying out and reporting of this research.

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# Study 4

Social housing conditions modulate the long-term increase in cocaine reward induced by intermittent social defeat.

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# Social Housing Conditions Modulate the Long-Lasting Increase in Cocaine Reward Induced by Intermittent Social Defeat

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Social defeat is considered the most representative animal model for studying the consequences of social stress. Intermittent social defeat (ISD) has proved to enhance the response to cocaine hedonic properties. In the present research, we evaluated if different social housing conditions, as housing with a familiar conspecific or with a female, exert a protective effect modulating the negative consequences of ISD as the increased sensitivity to cocaine and the induction of anxiety-like behavior. To achieve this objective, non-stressed or ISD OF1 male mice were divided into five different experimental groups according to their social environment: standard housing (four adult males per cage); male adolescent or adult in pairs (two males per cage); and adult males housed with a female for a short or long period (3 days vs. the whole duration of the study). Anxiety-like behavior was evaluated 19 days after the last episode of ISD using an elevated plus maze (EPM), and 24 h later the animals underwent a conditioned place preference paradigm (CPP) induced by a sub-threshold dose of cocaine (1 mg/kg). Following CPP, biological samples were taken to measure striatal levels of interleukin 6 (IL-6) and plasmatic levels of oxytocin (OT). Our results confirmed that ISD animals housed in standard condition displayed an anxious phenotype, developed CPP and had increased levels of IL-6 in the striatum. However, animals housed with a female or with a familiar male since adolescence did not develop CPP and were protected against the anxiogenic and neuroinflammatory potential of ISD stress. In the group of animals paired with a female throughout the experimental procedure, an increase in OT levels may have underlain this buffering effect, while the protective effect of being housed with a familiar male mouse seems to be related with a better resolution of the stress response. The present results expand our knowledge of the neurobiology of vulnerability to drug addiction and highlight the benefit of social support for recovery from the adverse effects of social stress.

**Keywords: social defeat, oxytocin, cocaine, conditioned place preference, IL-6, social environment**

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## INTRODUCTION

Drug addiction is a chronic disorder characterized by loss of control over the use of a substance and relapse during cessation attempts (Koob and Volkow, 2010; Volkow and Morales, 2015). The development of substance use disorder (SUD) is multifactorial, and the vulnerability to develop an addiction depends on a complex interplay between biological and environmental factors (Strickland and Smith, 2014).

Among environmental influences, social factors are powerful determinants of behavior and health status (Kessler et al., 2010; Ajonijebu et al., 2017). In this regard, there is a growing interest among researchers in studying the influence of social factors in addictive disorders (Neisewander et al., 2012). Although social stimuli can act as positive natural reinforcers that compete with drug reward, other social interactions can be highly challenging and become stressors (Heilig et al., 2016). For instance, social experiences with a negative affective valence (isolation or bulling in the workplace) are linked with higher rates of drug abuse and vulnerability to relapse after periods of detoxification (Sullivan et al., 2006; Niedhammer et al., 2010). On the other hand, positive social environments, such as strong family ties, involvement and attachment, are associated with lower rates of drug use and better prognosis during treatment (Stout et al., 2012; Litt et al., 2016).

Basic research with animal models using social and hierarchic status has highlighted the dual role of social factors in addiction. Animals living in social environments that provide access to socially rewarding experiences, such as sexual behaviors and pair bonding, are protected against drug-related behaviors (Beloate and Coolen, 2017; Rodríguez-Ortega and Cubero, 2018). For instance, a study carried out with socially housed rodents that acquired cocaine self-administration (SA) behavior and then experienced a forced period of abstinence showed a lower risk of displaying cue-elicited cocaine-seeking behavior than socially isolated animals that underwent the same experimental procedure (Thiel et al., 2010). Similarly, group-housed animals showed a lower risk of drug- or stress-induced reinstatement of cocaine conditioned place preference (CPP) in a former research carried out in our laboratory (Ribeiro Do Couto et al., 2009). On the other hand, social stressor experiences have repeatedly been reported to enhance the response to drugs, to escalate drug consumption and to promote relapse (see revision in Neisewander et al., 2012; Montagud-Romero et al., 2016). For example, early-life social stress experiences, like poor maternal care or maternal separation, have shown to increase ethanol and cocaine consumption in rats in different studies (Francis and Kuhar, 2008; Isengulova et al., 2009). Among all the paradigms that model social stress in rodents, such as social deprivation, social instability, and territorial and maternal aggression, social defeat is considered the most representative for studying the physiological and behavioral consequences (Neisewander et al., 2012; Hammels et al., 2015). This paradigm closely mimics the reality of subordinate vs. aggressor relations in humans (Björkqvist, 2001; Selten et al., 2013), and its ecological validity is widely demonstrated (Miczek et al., 2008). Also named the resident-intruder paradigm, it is based on the territorial attack of a resident male confronted with a conspecific intruder. In these agonistic encounters, residents and intruders demonstrate natural offensive and defensive behaviors, which allows researchers to study the short- and long-term behavioral and physiological consequences of social defeat stress. Overall, the scientific literature affirms that experiences of repeated or intermittent social defeat (ISD) enhance the unconditioned and conditioned rewarding responses to psychostimulant drugs and precipitate the reinstatement of drug seeking in the SA and CPP paradigms, while chronic social defeat produces the opposite effects, with animals displaying a decreased tendency to consume cocaine (see revision in Neisewander et al., 2012; Shimamoto, 2018).

Several neurobiological theories have been proposed to explain stress-induced vulnerability, including alterations of corticotrophin-releasing factor (CRF; Ferrer-Pérez et al., 2018b), dopamine neurotransmission system (Reguilón et al., 2017) and epigenetic forms of plasticity (Montagud-Romero et al., 2016; Ajonijebu et al., 2017). Recent studies suggest that inflammatory processes mediate the effect of ISD stress with regard to an enhanced drug response and anxiety-like behavior (Ferrer-Pérez et al., 2018a). Chronic social defeat and ISD promote the activation of the immune system and trigger a pro-inflammatory state characterized by increased levels of cytokines such as interleukin IL-1β or IL-6 (Wohleb et al., 2011, 2013, 2014; Hodes et al., 2014; Stankiewicz et al., 2015; Pfau and Russo, 2016; Ferrer-Pérez et al., 2018a), which has also been reported to compromise the integrity of the brain blood barrier (Rodríguez-Arias et al., 2017). Within the framework of this theory of neuroinflammation, some researchers have explored anti-inflammatory interventions as therapeutic targets in stress-related disorders, which have proven to be effective in reversing cognitive impairments, anxiety-like behavior and the enhancement in cocaine response induced by social stress (Pfau and Russo, 2016; Duque et al., 2017; Ferrer-Pérez et al., 2018a).

Positive social environments have been reported to have a protective effect on SUD development. Oxytocin (OT), is a neuropeptide that is released during physical contact and potentiates social behaviors (Carter, 2003). It might be central explaining the buffering effect of positive social environments as it has a direct effect reducing the activity of the hypothalamic-adrenal-axis (HPA) during stress response (Lee et al., 2009). Additionally, several studies have revealed anti-inflammatory and antioxidant properties of OT (Karelina et al., 2011; Yuan et al., 2016). In fact, it has shown to be effective in attenuating behavioral and physiological consequences of social stressors such as isolation, and has proven to be effective in reversing depressive and anxiety-like behaviors (Windle et al., 1997; Grippo et al., 2012). In the present research, we have analyzed if the long-lasting negative consequences of ISD on the anxiety-like phenotype and cocaine response can be reversed by different positive social housing conditions (e.g., pairing with a familiar conspecific or a female). Additionally, we aimed to determine if the

physiological mechanism that underlies this protective effect is linked to an anti-inflammatory effect of social intervention that could be mediated by the release of OT. Increasing our knowledge of how social context contributes to responses to drugs can lead to new avenues of drug prevention and treatment.

# MATERIALS AND METHODS

## Animals

A total number of 195 OF1 mice were supplied by Charles Rivers (France). The mice were divided into groups of 92 adults (42 days old) and 24 adolescents (21 days old). On arrival at the animal facility, the experimental mice were housed in groups of four in plastic cages (27 × 27 × 14 cm), with the exception of 48 animals (24 adults and 24 adolescents) that were housed in pairs. In addition to the experimental mice, 44 adult OF1 females were employed to provide female-paired housing. Finally, 35 adult male OF1 mice, to be used later as residents in the social defeat encounters, were housed individually in plastic cages (21 × 32 × 20 cm) for a month prior to the experiments in order to induce heightened aggression (Rodríguez-Arias et al., 1998).

Regardless of the experimental group to which they were assigned, all the animals were kept under the same conditions: constant temperature; a reversed light schedule (white light on 8:00–20:00 h); and food and water available ad libitum, except during behavioral tests. The experimental protocol was approved by an Institutional Review Committee for the use of animal subjects (Comité d'Ética d'Experimentació i Benestar Animal, number 2015/VSC/PEA/00168). 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. Every effort was made to minimize the animals' suffering and reduce the number of animals used.

## Drugs

For CPP conditioning, animals were injected intraperitoneally with a dose of 1 mg/kg of cocaine hydrochloride (Alcaliber Laboratory, Spain) dissolved in physiological saline (NaCl 0.9%) and adjusted to a volume of 0.01 ml/g of weight. This dose of cocaine was selected on the basis of previous CPP studies (Montagud-Romero et al., 2016; Ferrer-Pérez et al., 2018a,b) showing 1 mg/kg to be a sub-threshold dose for inducing CPP in adult animals without previous stress or drug experiences and housed in standard condition.

## Experimental Groups and Experimental Design

Mice were divided into different experimental groups (depicted in **Figure 1**) based on housing conditions. Next, half of the animals in each housing condition underwent an ISD, while the other half underwent a similar manipulation procedure without the experience of social defeat (nonISD). Subsequently, 19 days after the last social defeat, anxiety was evaluated in the elevated plus maze (EPM) test. One day later, the CPP procedure was initiated. Biological samples were taken after the CPP protocol on PND >89.

## Apparatus and Procedures

### Intermittent Social Defeat (ISD) Procedure

The ISD protocol followed in this study has been widely validated as a social stressor (Hodes et al., 2014; Hammels et al., 2015) and has been described in detail in previously published research by our group (Ferrer-Pérez et al., 2018a,b). Five days prior to initiation of the ISD protocol, aggressive residents were screened to confirm appropriate levels of aggressive behavior. The aggression test was performed in the home cage of the resident by placing an intruder adult OF1 mice in the cage for 3 min. Any resident mouse showing a latency to attack of over 3 min was withdrawn from the experiment.

The social defeat episodes consisted of three phases, each of which began by introducing the "intruder" (the experimental animal) into the home cage of the "resident" (the aggressive opponent) for 10 min. During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interaction and species-typical threats from the aggressive male resident, thus leading to instigation and provocation. The wire mesh was then removed from the cage to allow physical contact between the two animals for a 5-min period. In the third phase, the wire mesh was put in place again to separate the two animals for another 10 min while allowing social threats by the resident. Intruder mice were exposed to a different aggressor 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. In order to minimize physical wounding during social defeats, the 5-min direct encounters were interrupted if the intruder displayed a submissive supine posture for more than 8 s or if it was bitten by the aggressor more than 12 times. All agonistic encounters were videotaped to confirm social defeat. The nonISD groups followed the same protocol, but without the presence of a "resident" mouse: the mouse was placed in a new cage enclosed with a wire mesh for 10 min, after which the mesh was removed for 5 min and then returned for the last 10 min of each exploration session.

### Conditioned Place Preference (CPP)

The CPP protocol consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference. For place conditioning, we employed sixteen identical Plexiglas boxes with black and white equal sized compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm). In brief, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-min period was recorded. Mice showing a strong unconditioned aversion (less than 33% of the time spent in both compartments) or preference (more than 67%) for any compartment were excluded from the study.

In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of

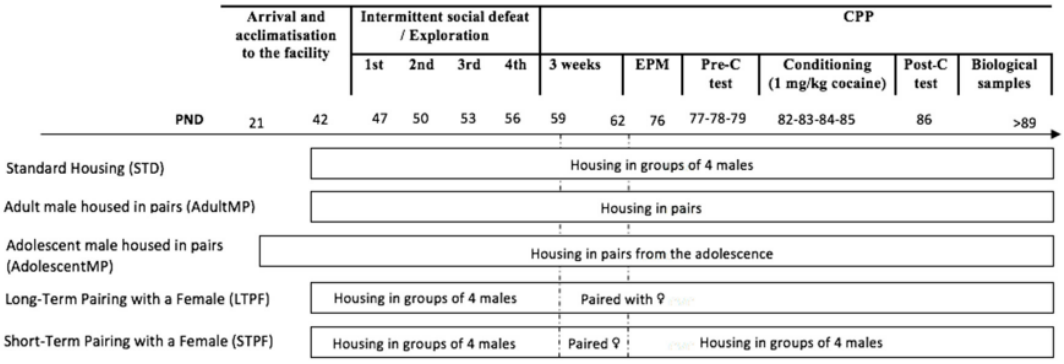


**FIGURE 1** | Experimental design. The experimental mice were divided into five different groups according to housing conditions. Animals in the Standard Housing group were housed in groups of four male mice per cage during the whole experiment (STD-nonISD $n$ = 12 and STD-ISD $n$ = 12). A second group of animals (AdultMP) were housed in pairs upon arrival at the animal facility on PND 42 (AdultMP-nonISD $n$ = 12 and AdultMP-ISD $n$ = 12). Similarly, a third group of adolescent mice (AdolescentMP) was housed in pairs on their arrival at the laboratory on PND 21 (AdolescentMP-nonISD $n$ = 12 and AdolescentMP-ISD $n$ = 12). Animals in the Long-Term Pairing with a Female (LTPF) housing condition were initially housed in the same way as those in the STD group, until the end of ISD protocol. Subsequently, 48 h after the last episode of social defeat, they were rehoused in a new cage with a female until the end of the experiments (LTPF-nonISD $n$ = 12 and LTPF-ISD $n$ = 12). Likewise, animals in the Short-Term Pairing with a Female (STPF) housing condition underwent the same housing procedure as LTPF animals, only that they were housed with the female for 72 h, after which, the experimental animals were returned to the original cage and regrouped as animals in standard housing groups (STPF-nonISD $n$ = 8 and STPF-ISD $n$ = 12).

physiological saline before being confined to the vehicle-paired compartment for 30 min. After a 4-h interval, they received cocaine immediately before being confined to the drug-paired compartment for 30 min. In the third phase (post-conditioning; Post-C) the conditioned preference was assessed by measuring the time spent by mice in a drug-free state in each compartment during the 15-min observation period. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is an estimation of the degree of conditioning induced by the drug. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the development of aversion. Additionally, a conditioning score (CS) was calculated for each mouse based on the difference between the time spent in the drug-paired compartment during the Post-C and Pre-C tests. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the induction of an aversion.

## Elevated Plus Maze-EPM

The EPM test was carried out essentially following the procedure described by Daza-Losada et al. (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, animals were habituated to the experimental room for 1 h prior to testing. At the beginning of each trial, experimental mice were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The behavior displayed by the mice during the test was recorded by an automated tracking system (EthoVision 3.1, Noldus) that tracks the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms were measured to characterize the anxiolytic effects of the different social housing conditions (Bourin et al., 2007; Blanco-Gandía et al., 2018).

## Tissue Sampling

Animals were sacrificed by cervical dislocation and then decapitated to collect blood from the neck in tubes coated with heparin. Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 min, 5,000 G) and transferred to sterile 0.2 ml microcentrifuge tubes. To obtain striatum samples brains were removed immediately after decapitation and dissected following the procedure described by Heffner et al. (1980). Plasma and tissue samples were stored at −80°C until IL-6 and OT determinations.

## Determination of Striatal IL-6 and Plasmatic Oxytocin Levels

To determine striatal IL-6 concentration we used a Mouse IL-6 ELISA Kit obtained from Abcam (Ref: Ab100712) following the manufacturer's instructions. Before running the kit, striatum samples were first homogenized and prepared following the procedure described in detail by Ferrer-Pérez et al. (2018a), and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

For the quantification of plasmatic OT, we used an ELISA kit from Arbor Assays (Ref: K048-H1). Following the recommendation of Leng and Sabatier (2016), we performed an extraction procedure to reduce the non-specific binding of plasmatic proteins in our samples. The extraction procedure was carried out using the extraction solution and the protocol provided by the ELISA kit manufacturer (Arbor Assays). ELISA test results were read using an iMark microplate reader

(Bio-RAD) controlled by Microplate Manager 6.2 software, and the final results were expressed in pg/mg for striatal tissue samples and in pg/ml for plasma.

## Statistical Analyses

A preliminary three-way analysis of variance (ANOVA) was carried out with the CPP data of animals under positive social housing condition with two between-subjects variables—Stress, with two levels (ISD and nonISD), and Housing, with four levels (AdultMP, AdolescentMP, LTPF, STPF)—and a within-subjects variable—Days, with two levels (Pre-C and Post-C). The preliminary statistical analysis carried out with the CPP data showed that animals housed in pairs with a female for a short (STPF) or a long term (LTPF) had equivalent results in this test. As a consequence, they were pooled in one single group (pairing with a female, PF) in further analyses. Additionally, the group of adult animals housed in pairs with other males (AdultMP) were removed from further experiment analyses as this intervention showed non-protective effects and both EXP and ISD animals displayed CPP. Outcomes of this group in each test are available as **Supplementary Material** (see **Supplementary Table S1**). Taking these results into account, subsequent ANOVAs included only three intervention groups: standard housed, adolescent male paired, and paired female.

A two-way ANOVA with the aforementioned between-subjects variables (Stress and Housing) was employed to analyze the data of EPM, IL-6, and OT levels. The value of the effect size was evaluated using partial eta-squared. Data are presented as mean ± standard error of the mean (SEM) and a $p$-value $< 0.05$ was considered statistically significant. Analyses were performed using SPSS v24. In all cases, *post hoc* comparisons were performed with Bonferroni tests.

# RESULTS

## Housing Conditions Decrease ISD-Induced Anxiogenic Behavior Evaluated in the EPM

The ANOVA of the EPM data (see **Figure 2**) revealed an effect of the interaction Housing × Stress on the time spent in the open arms ($F_{(2,86)} = 4.454$; $p = 0.014$; effect size 0.094) and on the percentage of open *entries* ($F_{(2,86)} = 4.304$; $p = 0.017$; effect size 0.091). ISD mice housed under the standard condition spent less time and less percentage of time in the open arms than their corresponding non-stress controls (nonISD; $p < 0.01$) and compared to animals housed with a female for long and short terms (PF; $p < 0.001$).

## Housing Conditions Modulate the Increase in the Cocaine-Conditioned Reward Induced by ISD Stress

The ANOVA performed for the CPP data (see **Figure 3A**) showed a significant effect of the interaction between the variables Days × Housing × Stress ($F_{(2,85)} = 3.198$; $p = 0.046$; effect size 0.070). As expected, ISD animals housed in the standard condition (STD-ISD) developed CPP, since they spent more time in the drug-paired compartment in the Post-C test than in the Pre-C test ($p < 0.01$). This time was also significantly higher when compared to the time spent in the drug-paired compartment by animals housed with a female for long and short terms (PF, $p < 0.05$).

The ANOVA for the CS (see **Figure 3B**) also revealed an effect of the variable Housing × Stress ($F_{(2,85)} = 3.235$; $p = 0.044$; effect size 0.071). Once again, socially stressed mice under standard condition housing (STD-ISD) had higher CS when compared to non-stressed animals in the same housing conditions (STD-nonISD, $p < 0.05$) and when compared to stressed animals housed with a female for long and short terms (PF, $p < 0.01$).

## The Neuroinflammatory Response Induced by Intermittent Social Defeat Stress Is Reduced by Positive Social Housing Conditions

A two-way ANOVA of IL-6 levels in the Striatum (see **Figure 4**) revealed an effect of the variable Housing ($F_{(2,66)} = 4.490$; $p = 0.015$; effect size 0.120). The *post hoc* test revealed that animals in STD housing condition had higher IL-6 levels than animals housed with other males since

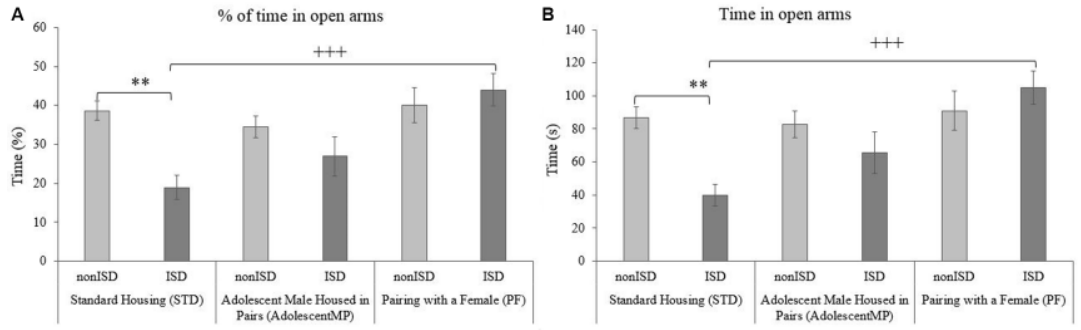


**FIGURE 2** | Positive housing conditions decrease ISD-induced anxiogenic behavior evaluated in the elevated plus maze (EPM). **(A)** Percentage of time spent in the open arms of the EPM. **(B)** Time (s) spent in the open arms of the EPM. $^{**}p < 0.01$ significant difference between STD-nonISD vs. STD-ISD. $^{+++}p < 0.001$ significant difference between STD-ISD vs. PF-ISD. Data presented as mean values ± standard error of the mean (SEM).

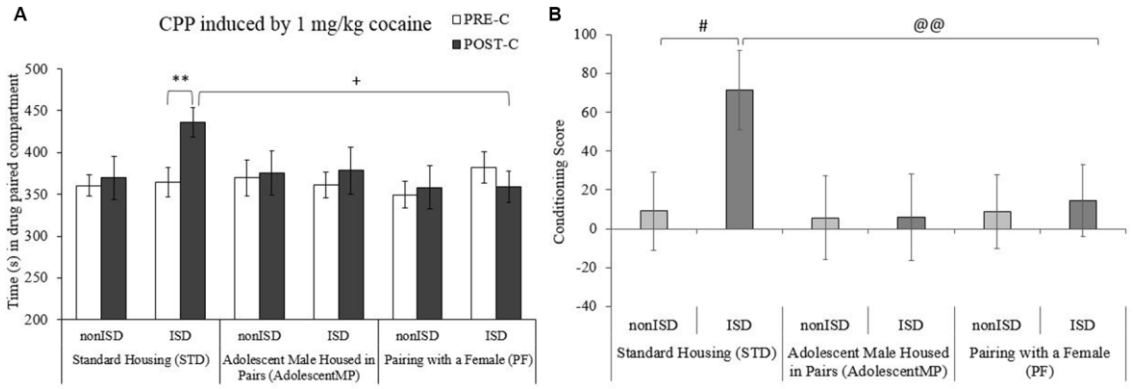


**FIGURE 3 |** Positive housing conditions decrease the effects of ISD stress on the acquisition of 1 mg/kg cocaine-induced conditioned place preference (CPP). Bars represent the following groups: standard housing (STD-nonISD $n = 12$; STD-ISD $n = 12$); male animals housed in pairs since adolescence (AdolescentMP-nonISD $n = 12$; AdolescentMP-ISD $n = 12$); male animals paired with a female for long or short term (PF-nonISD = 20, PF-ISD = 20). **(A)** The bars represent the time (s) spent in the drug-paired compartment in the PRE-C test (before conditioning sessions; white bars), and in the POST-C test (after conditioning sessions; black bars). Data are presented as mean values ± SEM. Bonferroni's test $^{**}p < 0.01$ significant difference in the time spent in the drug-paired compartment in POST-C vs. PRE-C in STD-ISD animals. $^{+}p < 0.05$ significant differences in the POST-C time between STD-ISD vs. PF-ISD. **(B)** Bars represent the conditioning score (CS), calculated as the time spent in the drug-paired compartment in the Pre-C test minus the time spent there in the Post-C test. $^{\#}p < 0.05$ significant difference between STD-nonISD vs. STD-ISD. $^{@@}p < 0.01$ significant difference between STD-ISD vs. PF-ISD.

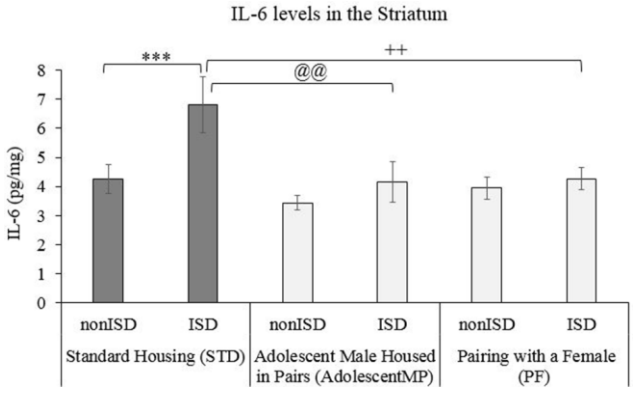


**FIGURE 4 |** Housing condition modulates the increase in IL-6 levels induced by ISD after CPP. Biological samples were obtained from animals in the following groups: standard housing (STD-nonISD $n = 12$; STD-ISD $n = 12$); Male animals housed in pairs since adolescence (AdolescentMP-nonISD $n = 8$; AdolescentMP-ISD $n = 8$); Male animals paired with a female for long or short term (PF-nonISD = 16, PF-ISD = 16). The bars represent the mean values of IL-6 striatal levels (pg/mg) ± SEM. Bonferroni test $^{***}p < 0.001$ significant difference in IL-6 levels between STD-ISD vs. STD-nonISD. $^{@@}p < 0.01$ significant difference in IL-6 levels between STD-ISD vs. AdolescentMP-ISD. $^{++}p < 0.01$ significant difference in IL-6 levels between STD-ISD vs. PF-ISD.

adolescence (AdolescentMP) or with a female (PF), $p < 0.05$ in both cases.

The ANOVA also revealed an effect of the variable Stress ($F_{(1,66)} = 7.809$; $p = 0.007$; effect size 0.106). ISD animals had higher striatal IL-6 levels when compared to the levels of non-ISD animals ($p < 0.01$).

Finally, the ANOVA also showed an effect of the interaction between Housing × Stress ($F_{(2,66)} = 3.266$; $p = 0.044$; effect size 0.090). Stressed animals under the standard housing condition (STD-ISD) had higher IL-6 striatal levels than non-stressed animals in the same housing condition (STD-nonISD; $p < 0.001$) than defeated animals housed in pairs since adolescence (AdolescentMP-ISD; $p < 0.01$) or those in the PF-IDS group ($p < 0.01$).

## Pairing With a Female (PF) Increases Plasmatic OT Levels

A two-way ANOVA of plasmatic OT levels (see **Figure 5**) revealed an effect of the variable Housing ($F_{(2,58)} = 3.154$; $p = 0.05$; effect size 0.098). *Post hoc* test revealed a significant increase of plasmatic OT levels in animals housed with a female for long or short terms (PF) when compared to those housed in the standard condition (STD; $p < 0.05$).

# DISCUSSION

The present research highlights how social factors are crucial in defining the individual's response to cocaine. Negative social events, such as agonistic encounters between conspecifics, are powerful stressors capable of altering physiologic and psychological functions. Animals that undergo four sessions of an ISD protocol display long-lasting alterations, including an anxious phenotype, enhanced sensitivity to the rewarding properties of cocaine, and increased pro-inflammatory signaling in the striatum. On the other hand, we have also seen that social enrichment in the form of positive housing conditions has a protective effect by reducing the above-mentioned negative consequences of social stress. Animals housed in positive social conditions—for instance, in pairs with a female or with a familiar male—are buffered against the

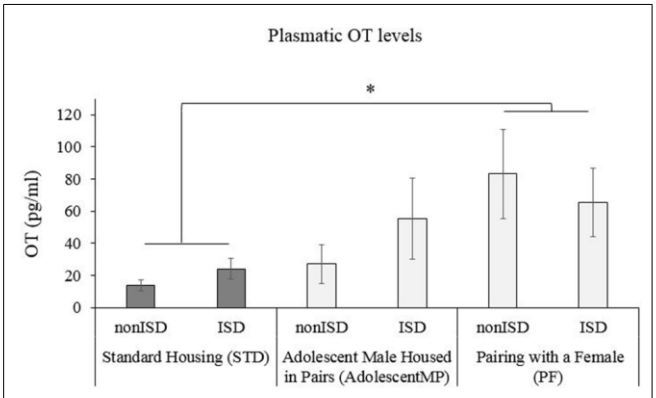


**FIGURE 5 |** Short and Long-Term Pairing with a Female (PF) increases plasmatic OT levels. The bars represent the plasmatic OT levels of mice in Standard Housing condition (STD-nonISD $n = 8$; STD-ISD $n = 8$); male animals housed in pairs since adolescence (AdolescentMP-nonISD $n = 8$; AdolescentMP-ISD $n = 8$); Male animals paired with a female for long or short term (PF-nonISD = 16, PF-ISD = 16). The bars represent the mean values of concentration (pg/ml) ± SEM. *$p < 0.05$ significant difference in OT levels compared to Standard Housing (STD) condition.

long-lasting increases in the rewarding properties of cocaine, anxiety-like behavior and the inflammatory response induced by ISD.

It is widely demonstrated that stressful social events have a modulatory effect on the effects of drugs (Gasparotto et al., 2005; Neisewander et al., 2012; Baracz et al., 2018). In our ISD protocol, the experimental mouse was confronted with a territorial (isolated) mouse that threatened and attacked the former, which adopted a defensive/submissive response (Miczek et al., 2008). Our results show that intruder animals experience these interactions as social stressors with a negative valence, and are subject to a series of long-lasting physiological and behavioral consequences that are consistent with previous evidence (Ferrer-Pérez et al., 2018a,b; Montagud-Romero et al., 2018). Socially defeated animals under standard housing condition (STD-ISD) showed increased sensitivity to the conditioned rewarding properties of cocaine, as they developed CPP for a subthreshold dose of cocaine (1 mg/kg), while the same dose was ineffective in animals that were not exposed to the ISD protocol (STD-nonISD). These animals also displayed an anxiety-like behavior phenotype characterized by spending less time in the open arms of the EPM than their non-stressed counterparts.

Rather than owing to a single mechanism, ISD effects are related with multiple and complex changes to peripheral and central systems that are not yet completely understood. Among these alterations, we hypothesize that immune response activation is critical in the appearance of the abovementioned long-lasting stress consequences. The results of the present study confirm this hypothesis by showing that socially defeated animals housed under standard condition (STD-ISD) display higher striatal levels of IL-6 than non-ISD animals. Following the same social defeat protocol, we have previously observed an increase in plasmatic and central levels of the pro-inflammatory cytokine IL-6 that returned to normal 3 weeks after the last defeat and increased again after cocaine-induced CPP (Ferrer-Pérez et al., 2018a). Therefore, we can affirm that intermittent social stress activates an initial immune response that promotes a sensitization of the neuroimmune axis and enhances the potential of cocaine to induce a pro-inflammatory state.

Previous studies indicate that positive social housing conditions are a successful intervention for reducing or preventing the negative consequences of social stress, mostly focus on anxiety behavior (Gasparotto et al., 2005; Nakayasu and Ishii, 2008; Neisewander et al., 2012). Housing with a female—thereby allowing mating behavior—for either long- (LTPF) or short-term (STPF) periods completely counteracted the anxiogenic effects of ISD and blocked cocaine-induced CPP, preventing the sensitization of the reward system by stress. Other researchers have reported that cohabitation with a female has a protective effect against the acquisition and extinction of cocaine CPP (Ribeiro Do Couto et al., 2009) and buffers against the anxiogenic effect of social and physical stress (Gobrogge and Wang, 2015). However, no studies have evaluated this cohabitation after exposure to social stress. We observed that this housing condition also exerted a protective effect against intermittent social stress-induced sensitization of the immune axis, as neuroinflammatory markers were not enhanced after the CPP procedure. We also observed that this housing condition induced a significant increase in plasmatic OT levels, which led us to suspect that this anti-inflammatory effect is mediated by the release of OT during social interaction in the cage. Although it is known that OT can exert an anti-inflammatory effect by decreasing the hypothalamic-adrenal-axis response to stressors (Lee et al., 2009; Karelina et al., 2011; Yuan et al., 2016), in our design, pairing with the female took place after the last defeat. Therefore, another mechanism that may explain the protective effect of this neuropeptide is that OT has the ability to change the focus from drug reward to social reward (McGregor and Bowen, 2012), thus enhancing the ability of a positive social stimulus to compete as an alternative reinforcer. In support of the role of OT in social defeat effects, we have recently reported that an injection of exogenous OT before each defeat episode induced a protective effect by blocking stress-increased anxiety-like behavior and the increased rewarding properties of cocaine in the CPP and the SA while favoring the extinction of drug memory (Ferrer-Pérez et al., 2019).

Free-living male mice prefer to live with females than with other males (Kappel et al., 2017). Group-housed male mice develop a social hierarchy, and under laboratory housing conditions it is common to witness inter-male aggression while dominance is established within the cage (Kappel et al., 2017). Indeed, aggressions can continue even after the establishment of a stable hierarchy, as a consequence of the alteration of territorial scent marking during the cleaning of cages (Poole and Morgan, 1973). Several research works have demonstrated that the status of an animal in the hierarchy of the cage modulates its vulnerability to the negative consequences of social stress. For instance, Yanovich et al. (2018) found that submissive animals are more likely

to display anxiety-like behaviors and enhanced attraction to addictive substances when exposed to stress, while dominant animals are more resilient to the negative consequences of stress. Considering the negative effects of the continuous fight for dominance that characterizes standard housing, we designed a low hierarchic stress housing condition as a buffer against the consequences of social defeat stress. We paired two adult males (AdultMP) with the intention of promoting a more predictable hierarchy and thus reducing the stress derived from fights to establish dominance. Previously, other researchers have reported that the strategy of housing two familiar rats together prevents anxiety-like behavior induced by social stress (Nakayasu and Ishii, 2008). Conversely to previous reports and to our predictions, this pairing of adult mice failed to prevent the negative effect of ISD stress, as the animals in question displayed a similar CPP for a subthreshold dose of cocaine to that registered in defeated animals housed in standard condition (STD-ISD). We should take into consideration that Nakayasu and Ishii (2008) employed a single episode of social defeat, while our protocol consisted of intermittent defeat encounters over several days. It is possible that, given that these housing conditions were established upon arrival of the animals at the laboratory (42 PND), and that social defeat or exploration protocols began immediately after the acclimatization period (on PND 47), animals were experiencing social defeat stress while the cage hierarchy was still being established; in this way, both animals in each pair would have been experiencing the dominance/submission stress that is usually resolved after 21 days (Poole and Morgan, 1973; Rodríguez-Arias et al., 1998). We believe that this initial stress during the early definition of hierarchic positions was less evident in the standard housing condition because of the size of the group (four mice). Not all four animals in standard housing conditions directly experienced the stress of the dichotomy of submissive/dominant roles, as there were animals that occupied an intermediate hierarchic position. To test this hypothesis, we repeated the experiment, but this time established the housing conditions during adolescence, on PND 21 (AdolescentMP), so that social defeat and the exploration protocol would take place after the initial instability of hierarchy establishment. Now, this housing condition became a protective environment, in line with our earlier predictions. The stress-enhanced response to the rewarding properties of cocaine and sensitization to the neuroinflammatory response induced by ISD were both blunted. In this case, the mechanism underlying the protective effect of being housed with a familiar male since adolescence was not directly related to the OT buffering. We hypothesize that the protective effect observed in the AdolescentMP group was the result of a better resolution of the stress response, which limits the negative consequences of social defeat stress that are secondary to maintenance of the cage hierarchy.

The present research highlights how social interactions with conspecifics are powerful mediators of the individual's response to drugs of abuse. All positive social housing conditions analyzed prevented the sensitization of the neuroimmune axis and the pro-inflammatory state induced by ISD. However, we were not able to prove the causal role of OT mediating this anti-inflammatory effect, as OT levels did not predict the variations observed in IL-6 concentration. Therefore, future research will be needed to identify the neurophysiological mechanism that underlies the buffering potential of positive social interactions against long-lasting ISD effects.

## ETHICS STATEMENT

The experimental protocol was approved by an Institutional Review Committee for the use of animal subjects (Comité d' Ética d'Experimentació i Benestar Animal, number 2015/VSC/PEA/00168). 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. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

CM, JM and MR-A designed, funded and administered the study. CM and JM reviewed the manuscript. CF-P, MR and MR-A designed, executed the study, analyzed the data, wrote and reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00148/full#supplementary-material

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**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Study 4

Endogenous oxytocin is essential for the buffering effects of social enrichment against the increase in cocaine reward induced by social stress.

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*In preparation*

**Endogenous oxytocin is essential for the buffering effects of social enrichment against the increase in cocaine reward induced by social stress**

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## 1. Introduction

This experiment was carried out based on previous studies developed in the present Doctoral Thesis. In study 3, we found that a pretreatment with exogenous oxytocin (OT) in a dose of 1mg/kg prior to repeated social defeat (RSD) episodes was an effective intervention in blocking the long-lasting increase in cocaine motivational properties and the anxious phenotype that social defeated animals display (Ferrer-Pérez et al., 2019a). Then, in study 4, we aimed to broaden our knowledge regarding the protective potential of the neuropeptide OT. The main objective of study 4 was to determine whether positive social housing conditions, an environmental intervention that has classically been proven to buffer stress consequences (Gasparotto, O et al., 2005; Nakayasu & Ishii, 2008; Neisewander, et al., 2012), exert its protective effect through oxytocinergic mechanisms. We evaluated the effect of different social housing modalities and found that social pairing was a protective housing condition counteracting the effects of social stress. Males housed with a familiar conspecific since adolescence and adult male mice housed in pairs with a female did not develop conditioned place preference (CPP) for a subthreshold dose of cocaine and were also protected against the inflammatory effects of RSD stress (Ferrer-Pérez et al., 2019b). When we tried to define the role of OT in this protective effect, we found that only animals housed in pairs with a female until the moment of sacrifice had higher plasmatic OT levels, while animals housed with another male or with a female for a short period of time had similar plasmatic levels than animals under non-socially enriched housing conditions. The lack of higher OT levels in mice housed with a female for a short time could be due to the long period of time between the housing condition and the OT determination. However, no changes were observed in males paired with an adolescent male during the whole procedure. Thus, endogenous OT levels were not a good predictor for the efficacy of social intervention, showing that the role of OT in social buffering is more complex than initially assumed.

The objective of the present study was to confirm whether the participation of endogenous OT is necessary for the buffering effects of positive social housing, specifically in the condition of housing with a familiar conspecific. Additionally, we aimed to determine if there is a critical period for this oxytocinergic modulation to exert its protective effects. For this purpose, we pharmacologically blocked OT activity by administering atosiban, an OT receptor antagonist that has been reported to counteract

physiological and psychological OT effects (Wang, et al., 2018). The antagonist was administered in two different moments of the experimental procedure: one group of animals received the antagonist 30 minutes prior to each social defeat episode, while the other group of animals received a daily injection of the antagonist for 21 days, beginning one day after the last episode of RSD.

## 2. Material and Methods

### 2.1. Animals

A total number of 102 OF1 male were purchased to Charles Rivers (France). A group of 24 mice arrived on PND 42 and were housed in groups of four in plastic cages (27 × 27× 14 cm) and the remaining 78 animals arrived at adolescent age (21 days old) and were housed in pairs. In addition to the experimental mice, 35 adult male OF1 mice were employed as resident aggressive mice in the social defeat encounters. Aggressors were housed individually in plastic cages (21 x 32 x 20 cm) for a month prior to the experiments in order to induce heightened aggression (Rodríguez-Arias et al., 1998).

Regardless of the experimental group to which they were assigned, all the animals were kept under the same conditions: constant temperature, a reversed light schedule (white lights on 8:00-20:00), and food and water available ad libitum, except during behavioral tests. The experimental protocol was approved by an Institutional Review Committee for the use of animal subjects (Comité d'Ética d'Experimentació i Benestar Animal, number 2015/VSC/PEA/00168). 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. Every effort was made to minimize the animals' suffering and reduce the number of animals used.

### 2.2. Drugs

The OT receptor antagonist atosiban was purchased to Sigma-Aldrich and administered daily to the corresponding animals in a dose of 1 mg/kg intraperitoneally (i.p.). For the CPP protocol, animals were administered i.p. with a dose of 1 mg/kg cocaine hydrochloride (Alcaliber laboratory, Spain). This dose of cocaine was selected on the basis of previous CPP studies (Ferrer-Perez e al., 2018; Montagud-Romero et al., 2016), showing 1 mg/kg to be a sub-threshold dose for inducing CPP in adult animals without

stress or drug experiences. Both drugs were dissolved in physiological saline (NaCl 0.9%) and adjusted to a volume of 0.01 ml/g of weight.

### 2.3. Experimental groups and experimental design

Animals were divided into different experimental groups depending on their housing condition: standard housing (SH) or housing in pairs (P); their stress condition: exploration (EXP) or repeated social defeat (RSD); and pharmacological treatment received: saline (SAL), acute atosiban (A.ATO) and chronic atosiban (C.ATO).

In the standard housing condition, adult mice were housed in groups of four. In the rest of experimental groups, adolescent (PND 21) animals were divided into pairs. Then, half of the animals in each housing condition started an RSD stress protocol on PND 47, while the other half underwent a similar handling but without experiencing social stress (EXP). Regarding the pharmacological treatment, animals in the EXP-P-A.ATO and RSD-P-A.ATO groups received an injection of the OT receptor antagonist 30 min before each exploration or social defeat episode. The remaining EXP-P-SAL, EXP-P-C.ATO, RSD-P-SAL and RSD-P-C.ATO animals did not receive any drug before social defeat or exploration episodes, but received one daily injection of saline or atosiban from the day after the last episode of social defeat until the day before the initiation of the CPP protocol. Subsequently, 19 days after the last social defeat, anxiety was evaluated in the elevated plus maze (EPM) test, and one day afterwards, the CPP procedure was initiated in all experimental animals. The experimental design and experimental groups are depicted in Figure 1.

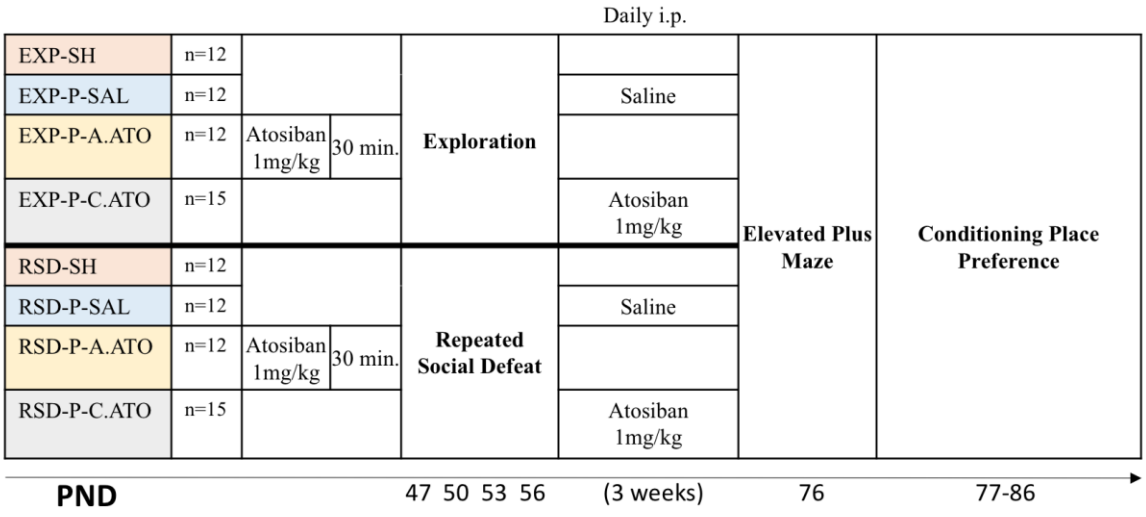


**Figure 1**. Experimental design.

### 2.4. Apparatus and procedures

**Procedure of repeated social defeat-RSD.** The social defeat protocol was carried out following the procedure previously validated and described in detail by Ferrer-Perez and coworkers (2018, 2019). Briefly, each experimental animal under the RSD condition experienced four episodes of social defeat. Each episode began by introducing the "intruder" experimental animal into the home cage of the "resident" (the aggressive opponent), which was protected by a wire mess wall for 10 minutes. During this initial phase, the experimental animal was protected from direct attack, exposed to social interaction and species-typical threats from the male aggressive resident. Then, the wire mesh was removed from the cage to allow confrontation between the two animals for a 5-minute period. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 minutes. 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 (Rodríguez-Arias et al., 1998). In order to minimize the physical wounding during social defeats, the 5-minute direct encounters were interrupted if the intruder displayed a submissive supine posture for more than 8 seconds or if it was bitten by the aggressor more than 12 times (Burke et al., 2016).

**Conditioned Place Preference-CPP**. The place conditioning protocol has been previously described in detail by Ferrer-Perez and coworkers (2018, 2019). This protocol consisted of three phases and took place during the dark cycle following an unbiased procedure in initial spontaneous preference terms. For place conditioning, sixteen identical Plexiglas boxes with equally sized black and white compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm) were used. In brief, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-minute period was recorded. Mice showing a strong unconditioned aversion (less than 33% of the time spent in both compartments) or preference (more than 67%) for any compartment were excluded from the study.

In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 minutes. After a 4-hour interval, they received cocaine immediately before being confined to the drug-paired compartment for 30 minutes. In the third phase

(post-conditioning; Post-C), the time spent by the untreated mice in each compartment during the 15-minute observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the development of aversion.

**Elevated plus maze-EPM.** The EPM test was carried out following the procedure described by Daza-Losada and coworkers (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm), two enclosed arms (30 × 5 × 15 cm) and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, animals were habituated to the experimental room for 1 hour prior to testing. At the beginning of each trial, the experimental mice were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 minutes. The behavior displayed by the mice during the test was recorded and tracked by an automated tracking system (EthoVision 3.1, Noldus) that analyzes the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms are measures generally used to characterize the anxiolytic effects of drugs or interventions (Blanco-Gandía et al., 2018; Bourin et al., 2007).

### 2.5. Statistical analyses

The establishment of CPP induced by cocaine was determined using a three-way ANOVA with two between subjects variables—Stress, with two levels (RSD and EXP) and Group, with 4 levels (SH, P-SAL, P-A.ATO and P-C.ATO)— and a within-subjects variable—Days, with two levels (Pre-C and Post-C). The EPM data was analyzed using a two-way ANOVA with two between subjects variables —Stress, with two levels (RSD and EXP) and Group, with 4 levels (SH, P-SAL, P-A.ATO and P-C.ATO).

Data are presented as mean ± SEM and a p-value $< 0.05$ was considered to be statistically significant. Analyses were performed using SPSS v24. In all cases, post-hoc comparisons were performed with Bonferroni tests.

## 3. Results

### 3.1. Chronic but not acute atosiban treatment reverted the protective effect of positive social housing against increased cocaine reward

Results from the CPP data are depicted in Figure 2. The ANOVA of the CPP data showed a significant effect of the interactions Stress x Group x Days [$F(3,93) = 2.674$, $p < 0.05$]. Post hoc analysis showed that socially defeated animals housed in standard condition (RSD-SH) developed CPP for a subthreshold dose of cocaine as they spent more time in the drug-paired compartment during Post-C when compared to the time spent in Pre-C ($p<0.001$). CPP was also recorded in socially stressed animals housed in pairs and treated daily with atosiban (RSD-P-C.ATO, $p<0.001$). This conditioned preference was not acquired by non-stressed animals (EXP) or stressed animals housed in pairs treated with saline (RSD-P-SAL) or acute atosiban (RSD-P-A.ATO).

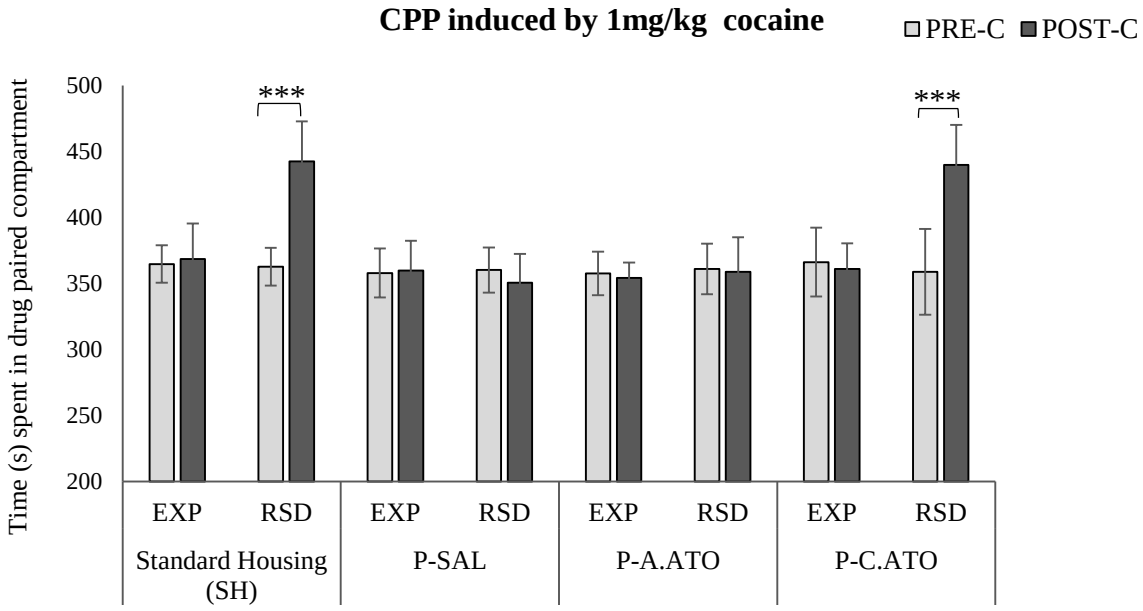


**Figure 2. Atosiban treatment reverted the protective effect of positive social housing.** Animals were divided into different experimental groups according to their housing condition: standard housing (SH) or housing in pairs (P); the stress condition: exploration (EXP) or repeated social defeat (RSD); and pharmacological treatment received: saline (SAL), acute atosiban (A.ATO) and chronic atosiban (C.ATO). Grey bars represent the time (s) spent in the drug-paired compartment in the PRE-C test (before conditioning sessions), and black bars represent the time in the POST-C test (after conditioning sessions). Data are presented as mean values ± SEM. Bonferroni's test ***$p<0.001$; significant difference in the time spent in the drug-paired compartment in the POST-C vs. PRE-C.

### 3.2. Acute or chronic treatment with the OT receptor antagonist atosiban did not modify the anxiolytic effect of positive housing conditions

The ANOVA of the EPM data (see Figure 3) revealed an effect of the interaction Stress x Group on the time [$F(3,105) = 2.858$; $p < 0.05$] and the percentage of time [$F(3,105) = 2.757$; $p < 0.05$] spent in the open arms. Post hoc analysis showed that socially stressed animals housed in standard condition spent less time and percentage of time in the open arms than non-stressed animals ($p<0.01$ in all cases).

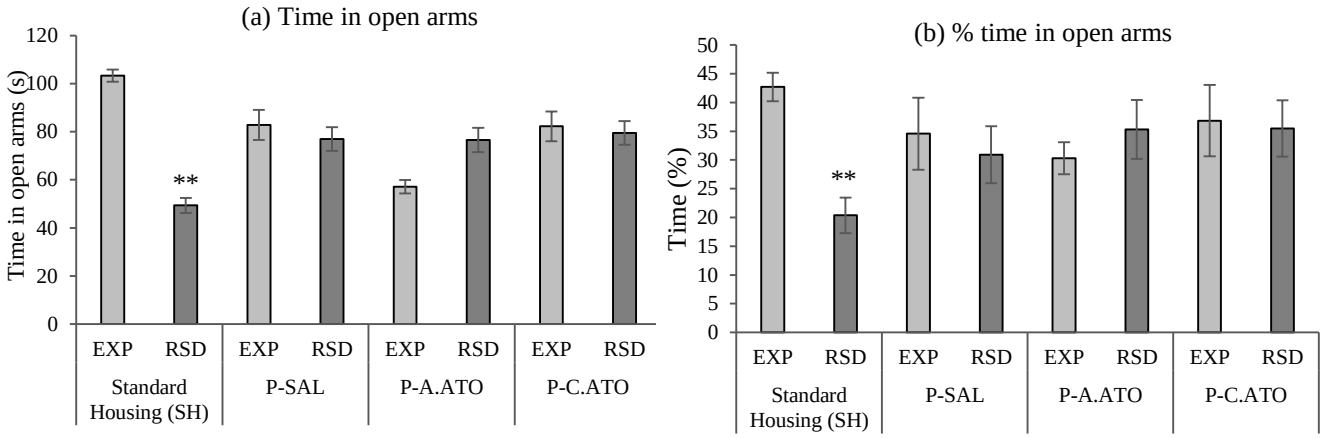


**Figure 3**. **Atosiban treatment did not modify anxiolytic effect of positive housing conditions**. Animals were divided into different experimental groups according to their housing condition: standard housing (SH) or housing in pairs (P); the stress condition: exploration (EXP) or repeated social defeat (RSD); and pharmacological treatment received: saline (SAL), acute atosiban (A.ATO) and chronic atosiban (C.ATO). (a) Time (s) spent in the open arms of the EPM. (b) Percentage of time spent in the open arms of the EPM. **p <0.01 significant difference vs. EXP-SH. Data presented as mean values ± SEM.

## 4. Discussion

Our results validate our previous findings that OT is crucial in mediating the protective effect of positive social housing conditions. Socially defeated animals housed in standard conditions (RSD-SH) displayed a long-lasting increase in the response to the rewarding properties of cocaine as they developed CPP induced by a subthreshold dose of the drug (1mg/kg) that was not effective in non-stressed animals. Moreover, RSD animals also displayed anxiety-like behavior evaluated in the EPM, as they spent less time and less percentage of time in the open arms of the maze compared to the time

spent by non-stressed animals. This result is consistent with previous experiments carried out in our laboratory and in other groups reporting that different protocols of social stress usually increase the response to rewarding properties of drugs of abuse and promote anxiety-like behavior (see revisions in Montagud-Romero et al., 2018; Shimamoto, 2018).

In the fourth study of this doctoral thesis, we found that being housed with a familiar male since adolescence was an effective environmental intervention buffering against social stress enhancement of cocaine reward and induced anxiety-like behavior (Ferrer-Pérez et al., 2019b). We have replicated these findings, as socially stressed animals housed in pairs (RSD-P-SAL) did not acquire CPP or display anxiety-like behavior. Moreover, chronic antagonism of OT receptors with 1mg/kg injection of atosiban reversed the abovementioned social buffering. Despite being housed in a protective environment, animals chronically administered with the OT receptor antagonist atosiban showed increased sensitivity to the conditioned rewarding properties of cocaine developing CPP. Maintained blockade of OT receptors with chronic atosiban administration after RSD experiences reversed the social protective effect, and animals displayed the classical enhanced response to cocaine expected in socially stressed animals. Some researchers have found that a decrease of OT endogenous neurotransmission amplifies neuroendocrine and the anxiogenic response to a stressor. For instance, Amico and coworkers (2004) reported that OT-deficient mice had an exaggerated response to a physical stressor with greater release of corticosterone. Moreover, prolonged social interactions seem to be required to promote sustained increases in OT levels (Faraji et al., 2018). In line with our results, these researchers found that a chronic pharmacological treatment (41 doses) with an OT antagonist (L-366,509) was effective in reversing the beneficial effects of social enrichment.

As we were already aware that an exogenous administration of 1mg/kg OT before RSD episodes reversed the sensitization of the reward system induced by social stress (Ferrer-Pérez et al., 2019a), we expected the acute antagonism of OT receptors prior to RSD stress to reverse the protective effect of positive social housing. However, acute treatment with atosiban 30 minutes prior to RSD episodes (RSD-P-A.ATO) did not interfere with the benefits of being housed in pairs.

Therefore, exogenous pharmacological increases of OT could exert its protective properties through its anti-inflammatory effect (Yuan et al., 2016). Thus, acute pretreatment could prevent the inflammatory response following social stress episodes. In fact, we have previously demonstrated that the administration of an anti-inflammatory drug (indomethacin) before each social defeat stress was an effective treatment counteracting the inflammatory consequence of RSD (Ferrer-Perez et al., 2018). Moreover, acute OT administration has been proven to exert a modulatory effect on pain response, having analgesic effects (Boll et al., 2018). All these effects combined may protect animals acutely treated with OT against the behavioral impact of RSD stress. The acute antagonism of endogenous OT levels only temporarily blocked OT analgesic and anti-inflammatory effects and, therefore, was not enough to the blunt social buffering effect. Moreover, sustained increased OT levels may promote the behavioral and epigenetic changes observed in animals housed under socially enriched environments. OT has been found to increase brain plasticity and stability of the epigenetic process (Gudsnuk & Champagne, 2012). Moreover, the benefit of these changes may be related to the affected brain area. Neuroplastic changes in brain areas related to social behavior have been reported to promote social contact and social reward (Wang et al., 2019). OT has been demonstrated to increase motivation to approach socially rewarding stimuli (Thirtamara-Rajamani et al., 2018). From this perspective, OT can shift the focus from drug reward to social reward (McGregor & Bowen, 2012), thereby enhancing the ability of a positive social stimulus to compete as an alternative reinforcer.

While the anxiolytic-like potential of exogenous OT in stressed rodents has been well established (see revision in Neumann & Slattery, 2016), we failed to demonstrate a participating role of endogenous OT in the anti-anxiety effect of being housed in pairs. Neither acute nor chronic treatment with the OT receptor antagonist blocked this effect, and none of the animals housed in pairs showed anxiety-like behavior in the EPM. In agreement with this finding, Babic and coworkers (2015) found that in chronically stressed mice, a constant antagonization of OT receptors did not increase stress-induced anxiety.

Therefore, our results show that, while endogenous OT activity is key in the social buffering against the enhancement in cocaine rewarding response, it may not participate in the anti-anxiogenic effect of social enrichment. One possible explanation is that OT's

anxiolytic effects, along with its effects to prevent the increase of cocaine reward, occur in different brain regions that are unevenly exposed to peripheral atosiban administration. As the blood brain barrier is not completely permeable to atosiban (McKee et al., 2007), some authors posited that the antagonist may exert central effects interacting only with the most permeable areas of the brain (Mak et al., 2012). While its anxiolytic effects are presumably related with its actions in the connections between the medial prefrontal cortex and the amygdala (Dayi et al., 2019), OT modulation of the reward system is related to its effect on dopaminergic neurotransmission in the ventral tegmental area and the nucleus accumbens (Hung et al., 2017). Differential permeability of the blood brain barrier in the structures of these brain pathways may explain whether atosiban has a central effect or not. Another possible explanation is that, in fact, anxiolysis promoted by social housing is not dependent on oxytocinergic neurotransmission. To discard the participating role of endogenous OT in the anti-anxiolytic of social enrichment, more experiments should be carried out evaluating the effects of a central OT antagonist.

With this study, we increased the understanding about OT's therapeutic potential in drug addiction and stress-related disorders. Through pharmacological and environmental modulations of the neuropeptide, we have demonstrated that OT is crucial for stress-buffering effects of positive social housing in enhanced drug response, while it was not so in mediating the anti-anxiolytic effects of this intervention.

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# Study 5

Environmental enrichment modulates the impact of social stress on the endocrine and immune systems and potentiates cocaine-induced conditioned place preference.

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*In preparation*

**Environmental enrichment modulates the impact of social stress on the endocrine and immune systems and potentiates cocaine-induced conditioned place preference**

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## 1. Introduction

In recent years, numerous preclinical studies have explored the interaction between environmental enrichment (EE) and drug-related behaviors (Galaj et al., 2017). Environmental conditions are powerful determinants of animal behavior and well-being. (McQuaid et al., 2018). In laboratory facilities, mice are housed under standardized conditions that contribute to more homogenous interlaboratory subject environments (Toth et al., 2011). These housing environments may limit animal opportunity to engage in species normal behaviors, promoting abnormal functioning and an increased risk to several disorders, such as stereotypes (Garner, 2005). Minimal environmental conditions that ensure animal health and welfare are legally stipulated within the European Union by Directive 2010/63/EU. More intensive environmental enrichment (EE) strategies have been used, such as experimental interventions (André et al., 2018), showing that nesting material and/or shelters are useful to improve animal welfare without compromising a study's validity and reproducibility (Bayne & Würbel, 2014). EE interventions are based on housing conditions that grant the animal higher sensory, motor, cognitive and social stimulation in comparison with standard housing conditions (Toth et al., 2011).

EE usually consists of social housing in larger cages with increased environmental complexity that promote physical activity through the interaction with items and materials that can be manipulated and used for species typical behaviors, such as hiding and nesting (McQuaid et al., 2018). These environments have been traditionally related to enhanced psychological and physiological well-being, increased cognitive functioning (He et al., 2017; Li et al., 2017) and improved brain recovery (McDonald et al., 2018). EE has also been reported to enhance stress resilience (Aujnarain et al., 2018; Benaroya- Milshtein et al., 2004), being effective in protecting against the physiological and behavioral maladaptive effects of chronic and acute stress (Bahi, 2017). For example, mice rehoused in EE conditions after physical stress (restrain) or social stress (chronic subordinate colony) were protected against the anxiolytic effects of these stress experiences (Ashokan et al., 2016; Bahi, 2017).

The attenuation of stress consequences is particularly interesting in drug-related disorders, as stress is considered a risk factor in the genesis and chronicity of this disorder (Koob, 2008; Koob & Schulkin, 2018). Experimental evidence has shown that

EE has both a preventive and a curative effect on different animal models of addiction (Solinas et al., 2010). Chauvet and coworkers (2009) trained rats to self-administrate cocaine and then forced abstinence from the drug. They found that EE reduced drug seeking and stress-induced reinstatement of cocaine self-administration behavior. In another experiment, EE introduced after a protocol of chronic social stress was an effective intervention in modulating the increase of ethanol intake and ethanol-induced conditioned place preference (CPP) (Bahi et al., 2017).

The general buffering effects of EE interventions against stress have been linked to its potential to stimulate functional changes and promote neuroplastic processes that include increased levels of brain-derived neurotrophic factor (BDNF), neurogenesis and synaptogenesis (Dandi et al., 2018; Monteiro et al., 2014) in different brain areas such as the hippocampus, the amygdala and the dopaminergic mesolimbic system (Ashokan et al., 2016; Chauvet et al., 2009; Novkovic et al., 2015; Solinas et al., 2008). Additionally, EE provides various forms of stimulation that may act as alternative reinforcers to drugs (Ranaldi et al., 2011).

The present study aimed to analyze if being housed in an enriched environment exerts a protective effect against acute and long-lasting physiological and behavioral consequences of social stress. Specifically, we evaluated if two different modalities of EE, one composed by carboard items and another composed by durable PVC items, exerted a buffering effect over the acute neuroendocrine response to social defeat and against long-lasting stress consequences such as induced anxiety-like behavior, enhanced inflammatory response and increased cocaine rewarding properties.

## 2. Material and Methods

### 2.1. Animals

A total number of 68 OF1 adult male mice (42 days old) were purchased to Charles Rivers (France). On their arrival to the laboratory, a group of 20 mice were housed in standard housing conditions in groups of four in plastic cages (27 × 27× 14 cm), and the remaining 48 were housed also in groups of four in bigger cages (59 x 38 x 20 cm) with two modalities of EE, one consisting of items made of cardboard and another one with PVC items. In addition to the experimental mice, 30 adult male OF1 mice were

employed as resident aggressive mice in the social defeat encounters. Aggressors were individually housed in plastic cages (21 x 32 x 20 cm) for a month prior to the experiments in order to induce heightened aggression (Rodríguez-Arias et al., 1998).

Regardless of the experimental group assigned, all the animals were kept under the same conditions: constant temperature; a reversed light schedule (white light on 8:00-20:00); and food (Teklad Global Diet 2014) and water available ad libitum, except during behavioral tests. The experimental protocol was approved by an Institutional Review Committee for the use of animal subjects (Comité d'Ética d'Experimentació i Benestar Animal, number 2015/VSC/PEA/00168). 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. Every effort was made to minimize the animals' suffering and reduce the number of animals used.

### 2.2. Drugs

For the CPP protocol, animals were administered i.p. with a dose of 1 mg/kg cocaine hydrochloride (Alcaliber laboratory, Spain). This dose of cocaine was selected on the basis of previous CPP studies (Ferrer-Perez e al., 2018; Montagud-Romero et al., 2016), showing 1 mg/kg to be a sub-threshold dose for inducing CPP in adult animals without stress or drug experiences. Both drugs were dissolved in physiological saline (NaCl 0.9%) and adjusted to a volume of 0.01 ml/g of weight.

### 2.3. Experimental groups and experimental design

Animals were housed in different housing set-ups that were established on the arrival to the laboratory and maintained until the end of the experimental procedure. The different experimental groups can be distinguished: standard housing (STD), housing in an enriched environment (EE) with cardboard items (EECardboard) or housing in an enriched environment (EE) with PVC items (EEPVC). Half of the animals in each housing condition started on PND 47 a repeated social stress protocol (RSD), while the other half underwent a similar handling but without experiencing social stress (EXP). Subsequently, 19 days after the last social defeat, anxiety was evaluated in the elevated plus maze (EPM) test, and one day afterwards, the CPP procedure was initiated in all

experimental animals. The experimental design and experimental groups are depicted in Figure 1.

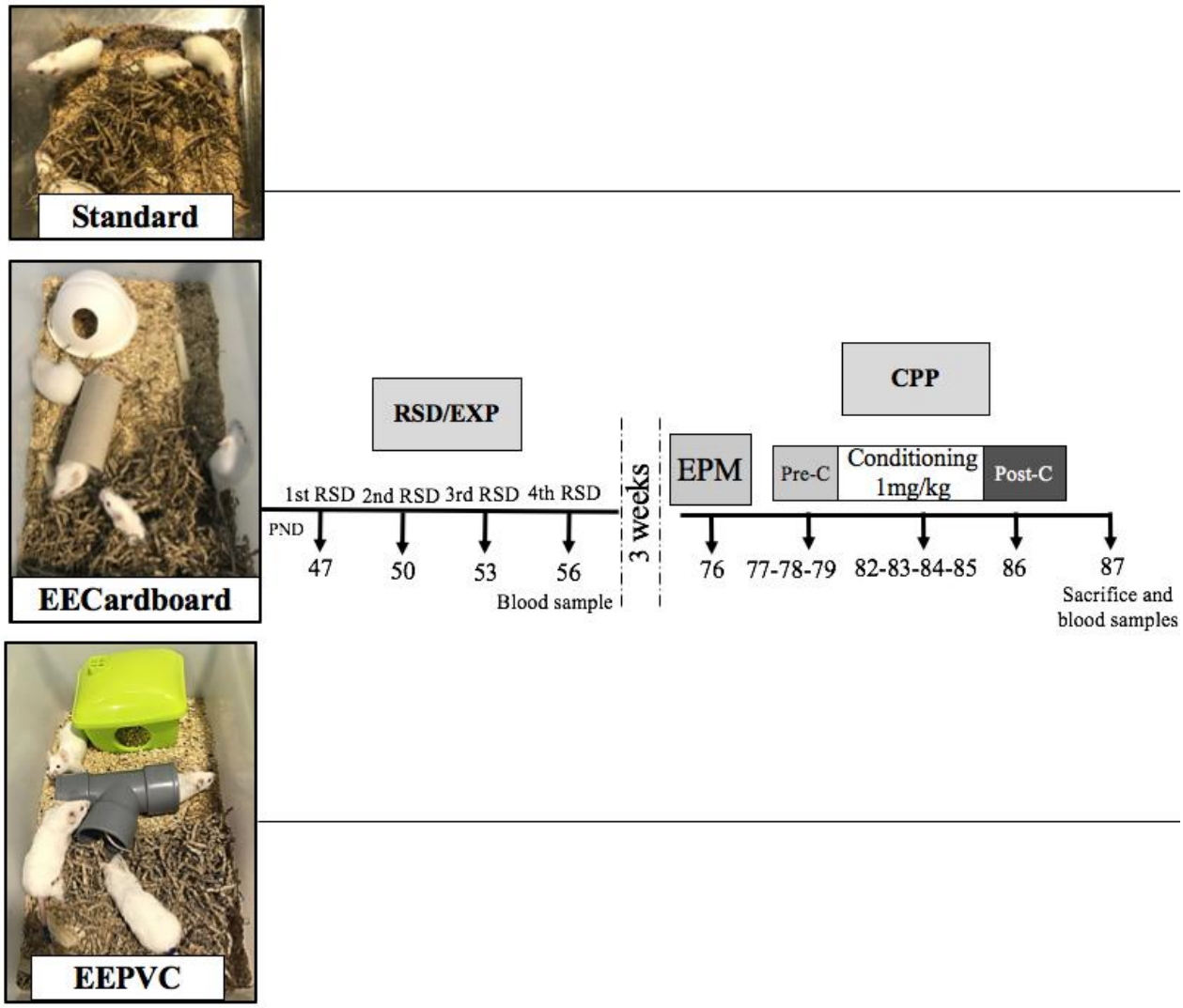


**Figure 1. Experimental design and experimental groups**

### 2.4. Apparatus and procedures

**Housing conditions.**

Standard housing (STD): animals were housed in groups of 4 in transparent plastic cages (27 × 27× 14 cm) with no more enrichment than standard bedding (wood flakes 1-3.35 mm), nesting material (paper strands) and two aspen gnaw sticks (5 x 1 x 1 cm) per cage.

Enriched environment (EE) with cardboard items (EECardboard): animals were housed in groups of 4 in plastic cages (59 x 38 x 20 cm) with standard bedding, nesting material and two aspen gnaw sticks plus an additional cardboard tunnel (9 × 4.5 cm) and a dome house (11 x 9 cm, Panlab). Cardboard enrichment items were changed every week for new ones.

Enriched environment (EE) with PVC items (EEPVC): animals were housed in groups of 4 in plastic cages (59 x 38 x 20 cm) with standard bedding and nesting material, two wooden gnaw sticks plus additional PVC tunnel (13 × 5.5 cm) and a plastic mouse house (12.5 x 10.5 x 11 cm).

**Procedure of social defeat.** Procedure of repeated social defeat (RSD). The social defeat protocol was carried out following the procedure previously validated and described in detail by Ferrer-Perez and coworkers (2018, 2019). Briefly, each experimental animal under the RSD condition experienced 4 episodes of social defeat. Each episode began by introducing the "intruder" experimental animal into the home cage of the "resident" (the aggressive opponent) protected by a wire mess wall for 10 minutes. During this initial phase, the experimental animal was protected from direct attack, exposed to social interaction and species-typical threats from the male aggressive resident. Then, the wire mesh was removed from the cage to allow confrontation between the two animals for a 5-minute period. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 minutes. 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 (Rodríguez-Arias et al., 1998). In order to minimize the physical wounding during social defeats, the 5-minute direct encounters were interrupted if the intruder displayed a submissive supine posture for more than 8 seconds or if it was bitten by the aggressor more than 12 times (Burke et al., 2016).

**Elevated plus maze-EPM.** The elevated plus maze (EPM) test was carried out essentially following the procedure described by Daza-Losada and coworkers (2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm) and a central platform (5 × 5 cm) elevated 45 cm above floor level. In order to decrease experimental stress, animals were habituated to the experimental room for 1 hour prior to testing. At the beginning of each trial, experimental mice were placed on

the central platform so that they were facing an open arm and were allowed to explore for 5 minutes. The behavior displayed by the mice during the test was recorded and tracked by an automated tracking system (EthoVision 3.1, Noldus) that analyzes the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). The time and percentage of time spent in the open arms are measures generally used to characterize the anxiolytic effects of drugs or interventions (Blanco-Gandía et al., 2018; Bourin et al., 2007).

**Conditioned Place Preference-CPP**. The place conditioning protocol has been previously described in detail in Ferrer-Perez and coworkers (2018, 2019). This protocol consisted of three phases and took place during the dark cycle following an unbiased procedure in initial spontaneous preference terms. For place conditioning, sixteen identical Plexiglas boxes with black and white equal sized compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm) were used. In brief, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-minute period was recorded. Mice showing a strong unconditioned aversion (less than 33% of the time spent in both compartments) or preference (more than 67%) for any compartment were excluded from the study.

In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 minutes. After a 4-hour interval, they received cocaine immediately before being confined to the drug-paired compartment for 30 minutes. In the third phase (post-conditioning; Post-C), the time spent by the untreated mice in each compartment during the 15-minute observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced by the drug. Additionally, a conditioning score was calculated for each mouse based on the difference between the time spent in the drug-paired compartment during the Post-C and Pre-C tests. If this difference is positive, then the drug is considered to have induced a preference for the drug-paired compartment, whereas the opposite indicates the development of an aversion.

**Biological samples obtaining**

Blood sampling for corticosterone determination was performed following the same procedure previously published in Montagud-Romero and cols (2017) and collected 30 min after the fourth episode of RSD. Using the tail-nick procedure, the animal was immobilized and wrapped in a cloth so as to decrease experimental stress, and through a 2-mm incision at the end of the tail artery, 50 µl of blood was collected. Blood samples were kept on ice and plasma was separated from whole blood by centrifugation (5 minutes, 5000G), transferred to sterile microcentrifuge tubes and stored at -80ºC until corticosterone determination.

Blood sampling for IL-6 determination was carried out 24 hours after finishing CPP procedure. Animals were sacrificed by cervical dislocation and then decapitated to collect blood from the neck in tubes coated with heparin. Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 minutes, 5000G) and transferred to sterile 0.2 ml microcentrifuge tubes.

**Determination of corticosterone and IL-6.** Before determining plasmatic corticosterone levels, samples were diluted with the reagents provided in the EIA kit obtained from Enzo Life (Ref: ADI-900-097) and analyzed following the manufacturer's instructions. For the assessment of plasmatic IL-6 concentration, we used a Mouse IL-6 ELISA Kit obtained from Abcam (Ref: ab100712), which was performed following the manufacturer's instructions.

ELISA test results were read using an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software and final results were expressed in in pg/ml.

**2.5. Statistical analyses**

The establishment of CPP induced by cocaine was determined using a three-way ANOVA with two between subjects variables – Housing, with 3 levels (STD, EECardboard and EE PVC), and Stress, with two levels (RSD and EXP)- and a within-subjects variable—Days, with two levels (Pre-C and Post-C). Conditioning score data, corticosterone levels, IL-6 levels and EPM data were analyzed using a two-way ANOVA with the two abovementioned between subjects variables –Housing and Stress-.

Data are presented as mean ± SEM and a p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS v24. In all cases, post-hoc comparisons were performed with Bonferroni tests.

## 3. Results

### 3.1. Environmental enrichment prevents the increase in corticosterone levels induced by social defeat

Data of the corticosterone concentration after the 4th SD episode is depicted in Figure 2. The ANOVA showed a significant effect of the interaction of the variables Stress x Housing [$F(2,47) = 7.146$, $p = 0.002$]. Post hoc analysis revealed that socially defeated animals housed in standard housing conditions (RSD-STD) had significantly higher corticosterone leves when compared to the plasmatic corticosterone levels of non-stressed animals under the same housing condition (EXP-STD, $p<0.001$), and when compared to socially stressed animals housed in enriched environments (RSD-EECardboard, $p<0.001$ and RSD-EEPVC, $p<0.01$).

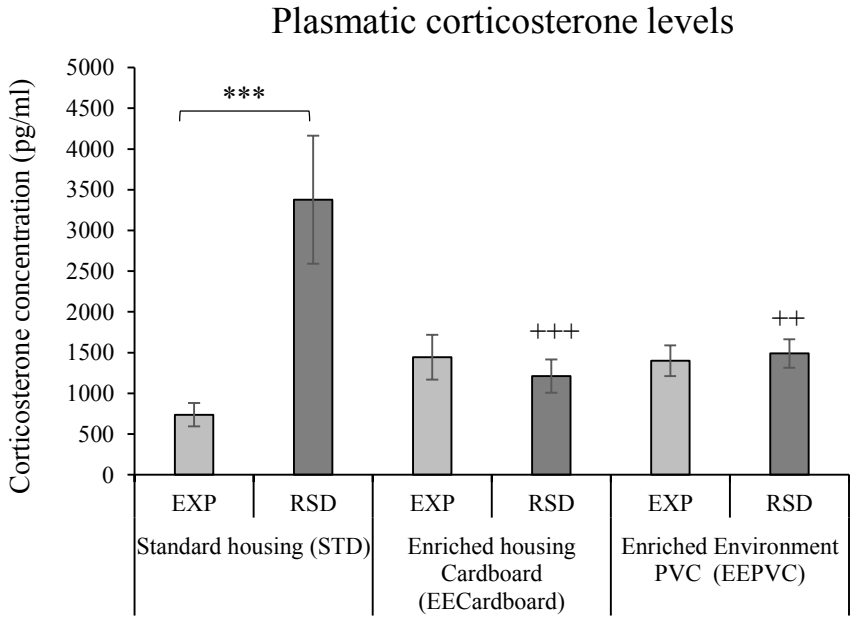


**Figure 2. Environmental enrichment prevents the increase in plasmatic corticosterone levels after an episode of social defeat.** Animals were randomly assigned to the following groups according to their housing and stress conditions: Standard housing (EXP-STD n=9; RSD-STD n=9); Housed in an enriched environment with board items (EXP-EECardboard n=7; RSD-EECardboard n=8); Housed in an enriched environment with PVC items (EXP-EEPVC n=7; RSD-EEPVC n=7). Data are

presented as mean values of corticosterone (pg/ml in plasma) ± SEM. Bonferroni's test ***p<0.001 significant difference in corticosterone levels EXP-STD vs. RSD-STD. ++p<0.01, +++p<0.001 significant differences in corticosterone levels vs. RSD-STD.

### 3.2. Environmental enrichment fails to prevent long-lasting anxiety-like behavior induced by social defeat

The ANOVA of the EPM data (see Figure 3) revealed an effect of variable Stress on the time spent in the open arms [$F(1,42) = 21.227$; $p < 0.001$] an in the percentage of time spent in open arms [$F(1,42) = 19.032$; $p < 0.001$]. Post hoc analysis showed that socially stressed animals spent less time and less percentage of time in the open arms than non-stressed animals ($p<0.001$ in both cases).

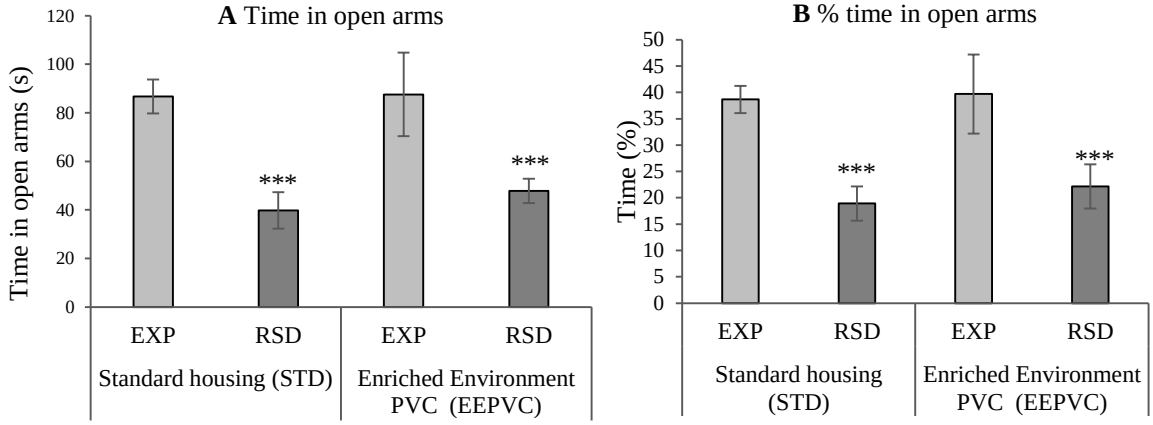


**Figure 3. Environmental enrichment fails to prevent long-lasting anxiety-like behavior induced by social defeat.** Animals were randomly assigned to the following groups according to their housing and stress conditions: Standard housing (EXP-STD n=12; RSD-STD n=12) and Housed in an enriched environment with PVC items (EXP-EEPVC n=10; RSD-EEPVC n=12). **A.** Time (s) spent in the open arms of the EPM. **B.** Percentage of time spent in the open arms of the EPM. ***p <0.001 significant differences between EXP vs. RSD. Data presented as mean values ± SEM.

### 3.3. Environmental enrichment increases the conditioned rewarding effects of cocaine

Results from the CPP data are depicted in Figure 4A. The ANOVA of the CPP data showed a significant effect of the variable Days [$F(1,61) = 40.638$, $p < 0.001$], and of the interaction of the variables Housing x Days [$F(2,61) = 5.046$, $p = 0.009$] and Housing x Days x Stress [$F(2,47) = 3.111$, $p = 0.05$].

Post hoc analysis showed that all experimental groups except non-stressed animals housed in standard conditions (EXP-STD) developed CPP for a subthreshold dose of cocaine, as they spent more time in the drug-paired compartment during the Post-C when compared to the time spent in the Pre-C: RSD-STD $p < 0.01$; EXP-EECardboard $p < 0.001$; RSD-EECardboard $p < 0.05$; EXP-EEPVC $p < 0.001$; RSD-EEPVC $p < 0.001$.

The ANOVA for the conditioning score (see Figure 4B) also revealed an effect of the variable Housing x Stress [$F(2,61) = 3.123$, $p = 0.05$]. All experimental groups showed higher conditioning than non-stressed animals in standard housing condition (EXP-STD): RSD-STD $p < 0.01$; EXP-EECardboard $p < 0.05$; RSD-EECardboard $p < 0.05$; EXP-EEPVC $p < 0.001$; RSD-EEPVC $p < 0.001$.

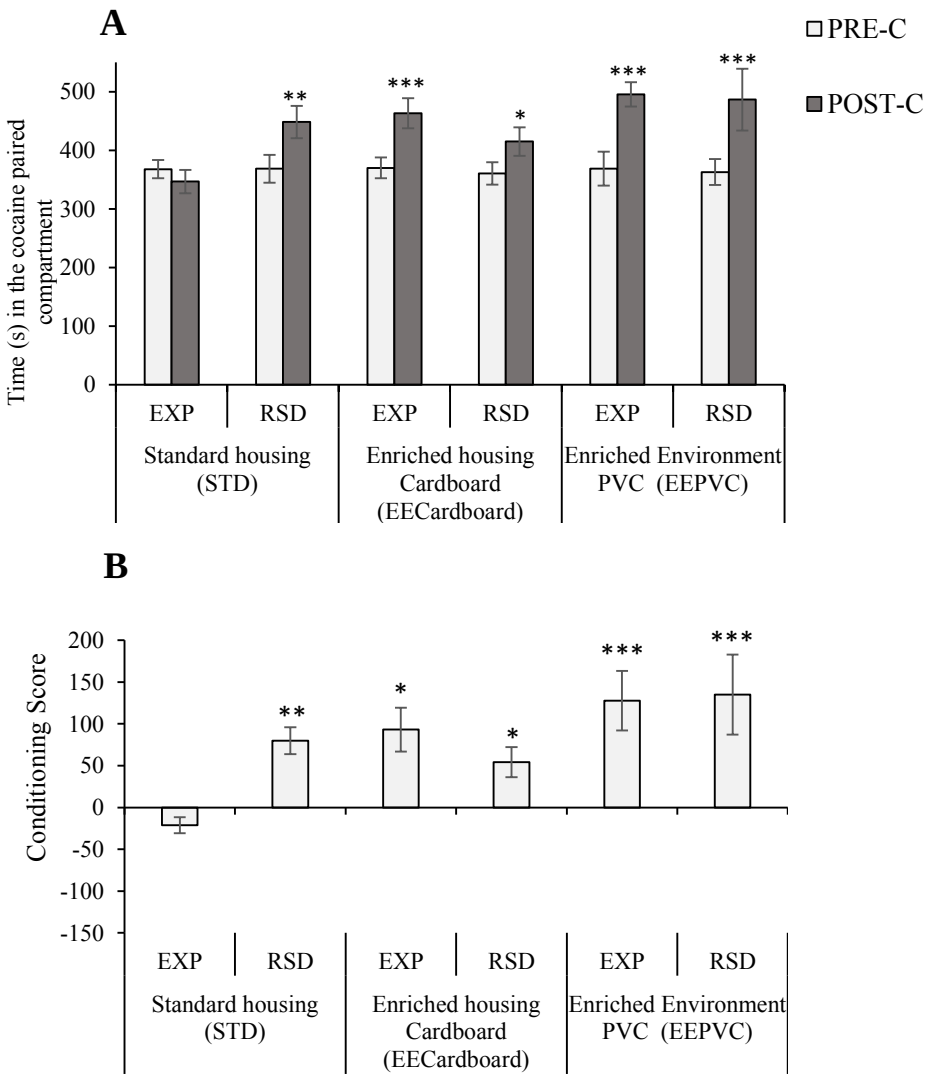

**Figure 4. Environmental enrichment increases the conditioned rewarding effects of cocaine** Animals were randomly assigned to the following groups according to their housing and stress conditions: Standard housing (EXP-STD n=10; RSD-STD n=10); Housed in an enriched environment with board items (EXP-EECardboard n=12; RSD-EECardboard n=12); Housed in an enriched environment with PVC items (EXP-EEPVC n=12; RSD-EEPVC n=12). **A.** Grey bars represent the time (s) spent in the drug-paired compartment in the PRE-C test (before conditioning sessions), and black bars represent the time in the POST-C test (after conditioning sessions). Data are presented as mean values ± SEM. Bonferroni's test; *p<0.05; **p<0.01; ***p<0.001 significant difference in the time spent in the drug-paired compartment in all animals in the POST-C vs. PRE-C. **B.** Bars represent the conditioning score (CS), calculated as the time spent in the drug-paired compartment in the PRE-C test minus the time spent there in the POST-C test. Bonferroni's test; *p<0.05; **p<0.01; ***p<0.001 significant difference in the CS between EXP-STD vs. RSD-STD, EXP-EECardboard; RSD-EECardboard; EXP-EEPVC and RSD-EEPVC.

### 3.4. Environmental enrichment modulates inflammatory response induced by RSD and cocaine CPP

Data of the IL-6 levels in plasma are represented in (Figure 5). The ANOVA showed a significant effect of the variable Stress [$F(1,36) = 28.25$, $p < 0.001$]; Housing [$F(2,36) = 7.877$, $p < 0.001$], and the interaction of the variables Stress x Housing [$F(2,36) = 5.695$, $p < 0.01$]. Post-hoc analysis showed that socially defeated animals under standard housing condition (RSD-STD) had higher IL-6 levels than non-stressed animals (EXP-STD) in the same housing condition ($p<0.05$). Similarly, socially defeated animals housed in an enriched environment with cardboard items (RSD-EECardboard) had higher plasmatic IL-6 levels than non-stressed animals in the same housing condition (EXP-EECardboard, $p<0.001$), and this levels were also significant higher when compared to RSD animals housed in standard conditions (RSD-STD) and with environmental enrichment of PVC items (RSD-EEPVC) ($p<0.001$ in both cases).

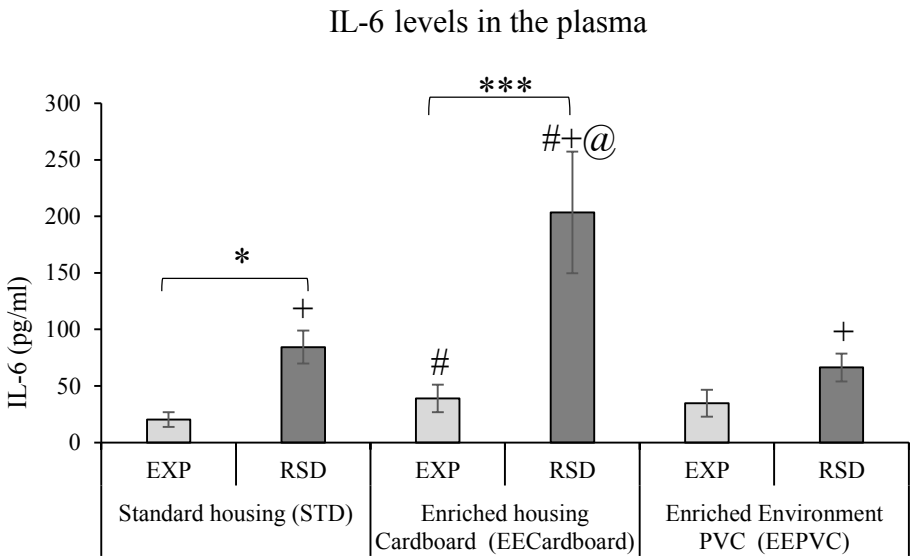


**Figure 5. Environmental enrichment modulates inflammatory response induced by RSD and cocaine CPP** Animals were randomly assigned to the following groups according to their housing and stress conditions: Standard housing (EXP-STD n=8; RSD-STD n=8); Housed in an enriched environment with board items (EXP-EECardboard n=7; RSD-EECardboard n=6); Housed in an enriched environment with PVC items (EXP-EEPVC n=7; RSD-EEPVC n=6). Data are presented as mean values of IL-6 (pg/ml in plasma) ± SEM. Bonferroni's test *$p<0.05$; ***$p<0.001$ significant differences of IL-6 levels between RSD-STD vs. EXP-STD and RSD-EECardboard vs. EXP-EECardboard. +$p<0.001$ significant differences of IL-6 levels between RSD vs. EXP. #$p<0.001$ significant differences of IL-6 levels between the group EECardboard vs. STD and EEPVC. @ $p<0.001$ significant differences of IL-6 levels between RSD-EECardboard vs. RSD-STD and RSD-EEPVC.

## 4. Discussion

In the present study, we found that EE effectively dampened the corticosterone response after social defeat, and in the condition of enrichment composed by plastic items, also prevented the sensitization of the immune system. However, conversely to our predictions, EE intervention enhanced cocaine potential to establish CPP, since all animals housed under enriched conditions developed CPP induced by a non-effective dose of the drug, regardless of stress exposure. In addition, anxiety-like behaviors characteristic of defeated mice were not counteracted by EE.

Our results confirmed that the social stress protocol (RSD) performed in the present study is an effective procedure to promote an endocrine stress response. We found that animals housed in standard condition presented a significant increase of plasmatic

corticosterone levels measured 30 minutes after the last agonistic encounter when compared to the hormone levels of animals that had undergone a similar experimental manipulation but that had not experienced social defeat (EXP). Interestingly, these elevations were not displayed by defeated animals housed in EE conditions, which had similar plasmatic levels to EXP mice. These results are consistent with previous reports where interventions based on EE strategies have proven to decrease the corticosterone response to stress (Benaroya-Milshtein et al., 2004; Dandi et al., 2018; McQuaid et al., 2012; 2018; Ravenelle et al., 2014). For instance, with a similar experimental design, McQuaid and coworkers (2012) recorded a remarked increase of corticosterone levels 75 minutes after an episode of social defeat on animals housed in standard condition and a reduction of this response among animals housed in an enriched environment. These results suggest that EE enhances the ability of mice to cope with stressors, potentially promoting a more adaptive HPA axis and a faster normalization of stress hormones (McQuaid et al., 2012; 2018).

However, even though EE exerted this clear buffering effect over the acute endocrine consequences of social defeat, it was not effective in counteracting the long-term anxiogenic effects of stress. We found that socially stressed mice, regardless of housing condition, displayed anxiety-like behavior in the EPM, as they spent less time and less percentage of time in the open arms of the maze compared to the time spent by non-stressed animals. Although EE interventions have been related to anxiolytic effects and to increasing resilience against anxiogenic effects of stress (Aujnarain et al., 2018; Benaroya-Milshtein et al., 2004; Lehmann & Herkenham, 2011), we did not find any effect of EE over an animal's performance in the EPM test. One possible explanation may be related to the moment of the developmental period in which the EE was introduced. McQuaid and coworkers (2012, 2018) found that if enrichment was introduced right after weaning, it exerted an anxiolytic effect, whereas if it was introduced to adult mice it exerted a clear anxiogenic effect evaluated in the EPM. Nevertheless, other factors should be underlying these discrepancies, as other researchers have reported anxiolytic effects of EE interventions even if they were introduced to adult mice (Ashokan et al., 2016).

There is a vast body of scientific publications that confirm the inflammatory effects of social defeat stress and its potential to induce a sensitization of the immune system to

future inflammatory challenges (Ambrée et al., 2018; Chan et al., 2019; Ferrer-Pérez et al., 2018). Moreover, the susceptibility of an individual to display pro-inflammatory states after stress experiences has been directly related to the susceptibility to develop stress-associated pathologies (see revision in Cathomas et al., 2019). We expected that EE intervention would have blocked the sensitization of the immune system induced by social stress. However, we did not record consistent results in this regard as we found that the two modalities of EE applied exerted differential effects. On the one hand, enrichment composed by items made of PVC plastic, which were maintained during the whole study, exerted a blunting effect over stress-induced immune sensitization. Socially stressed animals housed under these enriched conditions did not display an increase of plasmatic IL-6 levels in response to cocaine administration during CPP, as displayed by animals housed under standard condition. On the other hand, animals housed with carboard items showed the opposite pattern, since socially stressed animals displayed an exaggerated inflammatory response, even higher than that shown by defeated animals housed under standard conditions. While EE is widely confirmed to have an impact on the immune and inflammatory functions of rodents (Jurgens & Johnson, 2012; Kentner et al., 2016; Marashi et al., 2003; Pusic et al., 2016; Williamson et al., 2012), the valence of this modulation is still not clear. Some researchers have reported reductions in basal pro-inflammatory markers and diminished response to inflammatory challenges in animals housed in EE (Ali et al., 2019; Jurgens & Johnson, 2012; McQuaid et al., 2018; Silva et al., 2017; Williamson et al., 2012) while others have observed enhanced inflammatory responses (Marashi et al., 2003; McQuaid et al., 2012). An appealing explanation to these discrepancies was posited by Marashi and coworkers (2003). These researchers suggested that under certain circumstances, such as reduced space, EE interventions could enhance territorial aggression, promoting social stress and enhancing immune system reactivity. McQuaid and coworkers (2012, 2018) also found that aggressive behaviors mitigated the anti-inflammatory benefits of EE. They discovered that if EE was introduced in early stages of mice development, animals displayed low levels of aggression and reduced inflammatory response to stressors (McQuaid et al., 2018), but if EE was applied to adult mice, it increased inter-cage aggression and amplified inflammatory response to social defeat stress (McQuaid et al., 2012).

When conducting the experiment in the present study, we detected high levels of aggression in animals housed under EE with carboard items. Once a week, cardboard items were renewed, after which inter-cage aggressive behavior was witnessed. We believe that a constant changing of cage items disrupted territorial scent marking, a phenomenon that has been linked to enhanced aggression (Poole & Morgan, 1973). For this reason, we decided to include a new set-up of EE composed of PVC, which was durable and did not require periodic renewal. As expected, this strategy decreased aggressive behavior and, under these circumstances, a protective effect of EE over stress sensitization of the immune system was recorded.

Finally, we were not able to confirm a protective effect of EE intervention on long-lasting enhanced cocaine response induced by social stress. The dose of cocaine employed in this study (1mg/kg) is considered to be a non-effective dose to induce CPP in animals without previous experience with the drug or under homeostatic parameters (Montagud-Romero et al., 2018), but which becomes effective in sensitized animals, as we recorded in the present study in stressed mice housed in standard conditions. Unexpectedly, all animals housed in EE conditions displayed CPP induced by this subthreshold dose of cocaine. This result is not in agreement with previous studies, which mainly reported a protective effect of EE against drug-related behaviors (Nader et al., 2012), including a decreased response to the rewarding properties of drugs such as morphine (Xu et al., 2007), heroin (El Rawas et al., 2009) cocaine (Solinas et al., 2008) as well as a reduction of cocaine toxic effects (Freese et al., 2018). EE interventions have also been reported to be effective in decreasing the impact of stress over drug response, for instance Chauvet and coworkers (2009) found that a month of housing under EE conditions decreased cocaine-induced CPP and prevented cue- and stress-induced cocaine seeking reinstatement after extinction of drug memories. Nevertheless, our results are more in line with other studies that point that EE increases drug potential to induce CPP (Smith et al., 2009).

EE can induce an up-regulation of BDNF (Barros et al., 2019; Ickes et al., 2000; Meng et al., 2015) that has been linked to enhanced performance in spatial learning and memory tasks in rodents (Novkovic et al., 2015; Ohline et al., 2019). As CPP establishment is sustained by associative learning (Baruch et al., 2004) between the context and the conditioned reward induced by drugs, enhanced learning and memory

would impact the outcomes of CPP. In fact, Galaj and coworkers (2017) posited that some of the protective effects of EE over cocaine CPP, such as the reduction of the number of required sessions to extinguish the conditioned preference, are more related with a faster extinction of drug memories rather than a protective effect over the cocaine rewarding response itself. Thus, EE increases neuroplasticity and enhances associative learning, promoting more potent drug-associated memories. It also accelerates the extinction of drug-context memories, promoting the learning of the fact that the drug reward is no longer present.

In conclusion, in agreement with previous scientific reports, we found a buffering effect of EE intervention over some physiological consequences of social defeat stress, such as corticosterone response and long-term sensitization of the immune system, effect that only appeared if the inter-cage aggressive behavior was controlled. However, we were not able to demonstrate a protective effect on cocaine response. In fact, we found that EE experiences before CPP enhanced associative drug memories, promoting the establishment cocaine place preference. This potential of EE to enhance associative learning turns EE interventions into appealing research targets to explore interventions that favor the extinction of drug memories during drug cessation. Further studies with different time frames of EE exposure should be conducted in order to use EE-increased neuroplasticity as a beneficial tool to counteract stress detrimental effects.

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# Study 6

Effect of voluntary wheel running exercise on the endocrine and inflammatory response to social stress and in the sensitivity to the conditioned rewarding effects of cocaine.

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*In preparation*

**Effect of voluntary wheel running exercise on the endocrine and inflammatory response to social stress and in the sensitivity to the conditioned rewarding effects of cocaine**

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## 1. Introduction

Addiction can be defined as a chronic brain disorder originated by an interplay between biological and environmental factors. Stress is a central factor in the genesis of this disorder, with social adverse events being experienced more intensely than any other type of stressors (Almeida, 2005). Epidemiological studies show that negative social experiences such as isolation or bullying in the workplace are linked to higher rates of drug abuse and higher vulnerability to relapse after periods of detoxification (Niedhammer et al., 2010; Sullivan et al., 2006). Similar results have been recordered in basic research with rodent models. Social defeat, which is considered one of the most representative models in the study of psychophysiological consequences of stress (Hammels et al., 2015; Neisewander et al., 2012), has repeatedly confirmed to enhance sensitivity to the rewarding properties of different drugs (Montagud-Romero et al., 2018). To explain this stress effect on drug response, researchers have classically focused on studying the neurobiological adaptations in the neuroendocrine axis of stress and within the reward pathway. More recently, the mediation of inflammatory processes in stress-induced vulnerability to drug addiction has also been explored (Andersen, 2018; Ferrer-Pérez et al., 2018; Ganguly et al., 2019; Rodríguez-Arias et al., 2018).

One of the interventions that has been proven to be useful in buffering against the negative consequences of stress as well as in treating substance use disorders is physical exercise (Salmon, 2001). Physical exercise has a positive effect in the management of clinical stress (Souza Moura et al., 2015), increasing the ability to cope with daily stressful events without using drugs such as alcohol (Medina et al., 2011). Likewise, regular physical activity appears to have protective effects on the initiation of tobacco and other drugs during adolescence and early adulthood (Lisha & Sussman, 2010). Additionally, physical activity is also an interesting adjunctive intervention for drug cessation, with higher evidence demonstrated in tobacco treatment (Bardo & Compton, 2015; Linke & Ussher; 2015). However, there is not empiric evidence that proves a causal effect in humans (Lespine & Tirelli, 2018). In this regard, animal models allow for a better control of contaminating variables, such as socioeconomic factors, and a better manipulation of the independent variable regulating the duration and intensity of the exercise.

Voluntary wheel running (VWR) in rodents is widely used to mimic physical aerobic training in humans (Mul et al., 2018). In general, VWR has a protective effect in decreasing the endocrine and behavioral consequences of stress, inducing anxiolytic and antidepressants effects (Greenwood et al., 2003, 2011; Lynch et al., 2019). VWR also has a modulatory effect in all stages of the addictive cycle (Bardo & Compton, 2015; Lynch et al., 2013). During initial contact with drugs, access to the running wheel placed inside the homecage decreases voluntary consumption of ethanol in mice (Darlington et al., 2016; Ehringer et al., 2009). In the same line, animals with access to an activity wheel consume less of the drug during the acquisition of the self-administration behavior of methamphetamine, cocaine and heroin (Smith et al., 2008; Smith & Pitts, 2013; Smith & Witte, 2012). After the establishment of self-administration behavior, access to activity wheels has a protective effect in decreasing cue- and drug-induced reinstatement of cocaine administration (Smith et al., 2012) and nicotine seeking (Sanchez et al., 2013). On the other hand, results obtained using the conditioned place preference (CPP) paradigm, which evaluates secondary motivational properties of the drug (Tzschentke, 2007), are not so consistent (Lespine & Tirelli, 2019; Smith et al., 2008). Some studies point to the fact that animals with access to running wheels had lower cocaine or morphine-induced CPP compared to sedentary counterparts (Lett et al., 2002; Rozeske et al., 2011). However, other studies report no effects (Lespine & Tirelli, 2019) or an enhancement of the rewarding potential of morphine (Eisenstein & Holmes; 2007) and cocaine (Smith et al., 2008), even with an increased duration of drug-associated memories (Mustroph et al., 2011). Therefore, although physical activity is an appealing intervention to manage negative stress consequences, under certain circumstances it can be ineffective or increase stress effects (Lynch et al., 2013).

In order to increase the understanding about which physiological mechanisms underlie the protective effect of physical activity or can enhance stress effects, in the present paper we have compared the impact of four modalities of physical exercise (sedentary activity, housing with one or four voluntary running wheels and a regimen of intermittent voluntary wheel running) in the increase of cocaine conditioned reward induced by social stress. In addition, we have explored different neurobiological mechanisms that could be underlying the observed effects of physical exercise. (1) As there is evidence showing that social stress can induce inflammatory states that are

mediating the vulnerability to stress-related disorders (Cathomas et al., 2019; Hodes et al., 2014) and drug addiction (Ferrer-Pérez et al., 2018), we evaluated whether physical exercise could exert a modulatory effect over this stress-induced inflammatory response. (2) We also evaluated the potential of physical exercise to increase the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus and within the striatum, as these structures are key in the associative learning involved in CPP (Baruch et al., 2004) and plastic changes in these areas can sustain enhanced drug learning, as reported in previous studies (Eisenstein & Holmes; 2007; Greenwood et al., 2011). (3) We additionally measured corticosterone levels after social defeat stress episodes in order to determine a possible blunting effect of VWR in the neuroendocrine response of the stress. (4) Finally, we evaluated whether VWR can act as a hedonic substitute that competes with the rewarding potential of cocaine in the establishment of conditioned drug memories. The current work would increase the knowledge about the neurobiological mechanism that underlies the effect of physical exercise over the consequences of stress on drug response. This would help to optimize the designs of physical interventions, helping to choose the most beneficial regimen of exercise for the patient.

## 2. Material and methods

### 2.1. Animals

A total number of 146 OF1 adult mice (42 days old) were acquired from Charles Rivers (France). On arrival at the animal facility, a total number of 92 animals (sample for Study 1) were housed in plastic cages (59 x 38 x 20 cm) with one (1x4 Housing), four (4x4 Housing) or any (Sedentary Housing) voluntary running wheels. Another set of 54 animals (Study 2) were housed in groups of four in plastic cages (27 × 27× 14 cm) without additional environmental enrichment rather than nesting material. In addition to the experimental animals, 50 OF1 adult male mice were used as residents for the social defeat encounters.

Regardless of the assigned experimental group, all the animals were kept in the same conditions: constant temperature; a reversed light schedule (white light on 8:00-20:00); and food and water available ad libitum, except during behavioral tests. The experimental protocol was approved by an Institutional Review Committee for the use

of animal subjects (Comité d'Ética d'Experimentació i Benestar Animal, number 2015/VSC/PEA/00168). 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. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

### 2.2. Drugs

For CPP conditioning, animals were injected intraperitoneally with a dose of 1 and 3 mg/kg of cocaine hydrochloride (Alcaliber laboratory, Spain) dissolved in physiological saline (NaCl 0.9%) and adjusted to a volume of 0.01 ml/g of weight. These doses of cocaine were selected on the basis of previous CPP studies, showing 3 mg/kg cocaine is an effective dose to induce CPP in OF1 male mice (Reguilon et al., 2017) while 1 mg/kg is considered to be a sub-threshold dose CPP in adult animals without stress or drug experiences (Ferrer-Pérez et al., 2018).

### 2.3 Experimental groups and experimental design

In Study 1 (depicted in Figure 1), the effects of being housed with continuous or intermittent access to voluntary running wheels in the long-term effects of social defeat was evaluated. Upon arrival, animals were housed in groups of four in cages containing any (n=48); one (1x4, n=24) or four (4x4, n=20) activity wheels placed inside their home cage. Within the group of animals housed without activity wheels we could distinguish between the animals belonging to the sedentary condition (SED, n=24) with no additional physical activity, and the animals that followed an intermittent exercise regimen since the first week upon arrival and until the end of the study (INT, n=24). This regimen of intermittent exercise consisted of three sessions per week of 1 hour of individual access to a cage with a voluntary running wheel. Five days after the arrival and habituation to the animal facility, half of the mice in each experimental condition underwent a repeated social defeat (RSD) stress protocol (SED-RSD n=12; 1x4-RSD, n=12; 4x4-RSD, n=10; INT-RSD, n=12), while the other half underwent the exploration protocol (EXP), which involves a similar manipulation but without experiencing social defeat stress (SED-EXP, n=12; 1x4-EXP, n=12; 4x4-EXP, n=10; INT-EXP, n=12). On PND 56, 30 minutes after the last episode of social defeat or EXP, blood samples were taken in order to evaluate corticosterone levels. Subsequently, 21 days after the CPP

procedure induced by 1mg/kg was initiated. Biological samples were taken 24 hours after confirmation of the extinction of cocaine conditioned place preference.

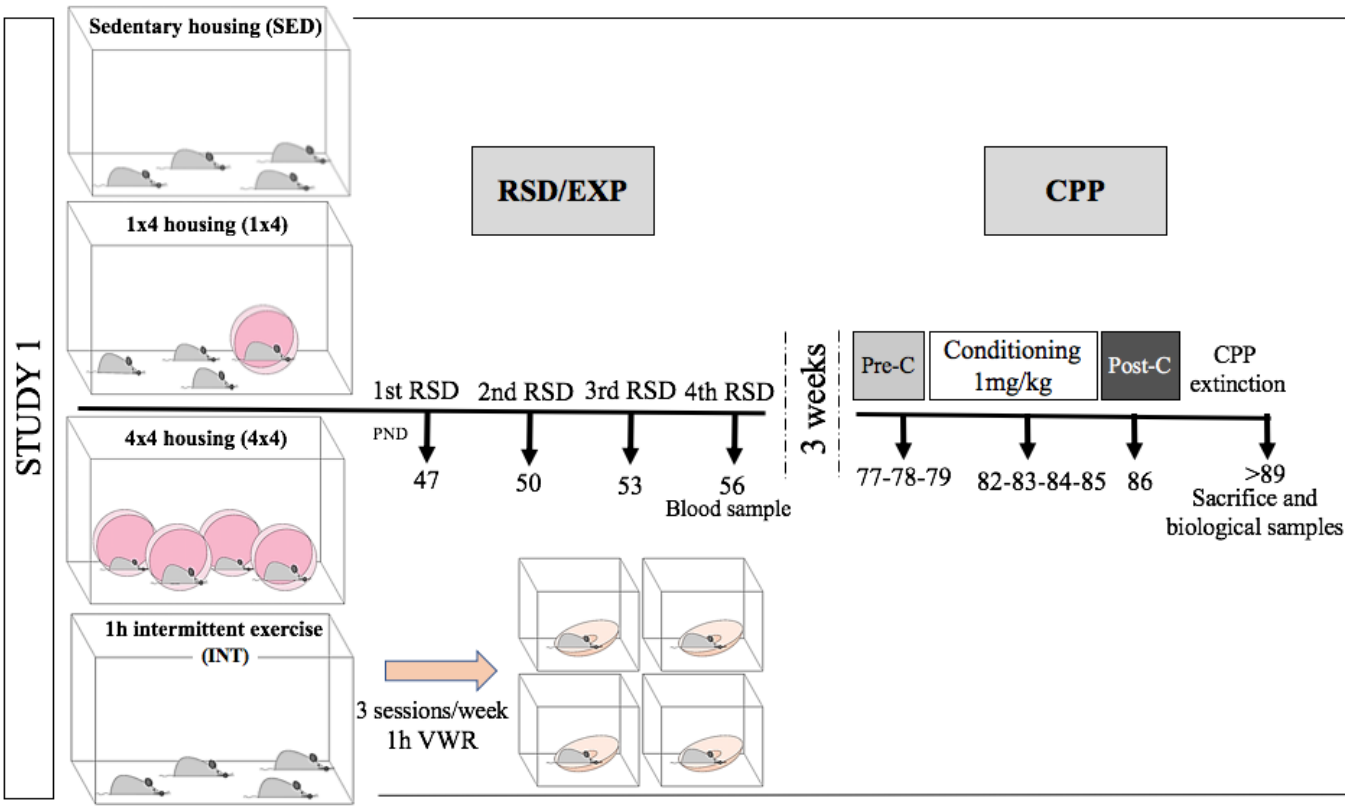



**Figure 1. Experimental design and experimental groups of Study 1**

In the second study (depicted in Figure 2), the rewarding potential of VWR to establish a CPP and to compete against cocaine reinforcement was analyzed. One group of animals underwent a traditional CPP protocol with the pairing of a dose of 3mg/kg cocaine (pictured in orange) with one compartment and an injection of saline (pictured in blue) in the other (3mg/kg-cocaine, n=15); a second group of animals (VWR group, n=21) experienced a CPP protocol in which an active running wheel was paired to one compartment and an inactive wheel was paired with the opposite compartment. Finally, in the third group (3mg/kg-cocaine-vs-VWR, n=17), an active running wheel and a saline injection was paired with one compartment while a dose of 3mg/kg cocaine and an inactive wheel was paired to the other compartment.

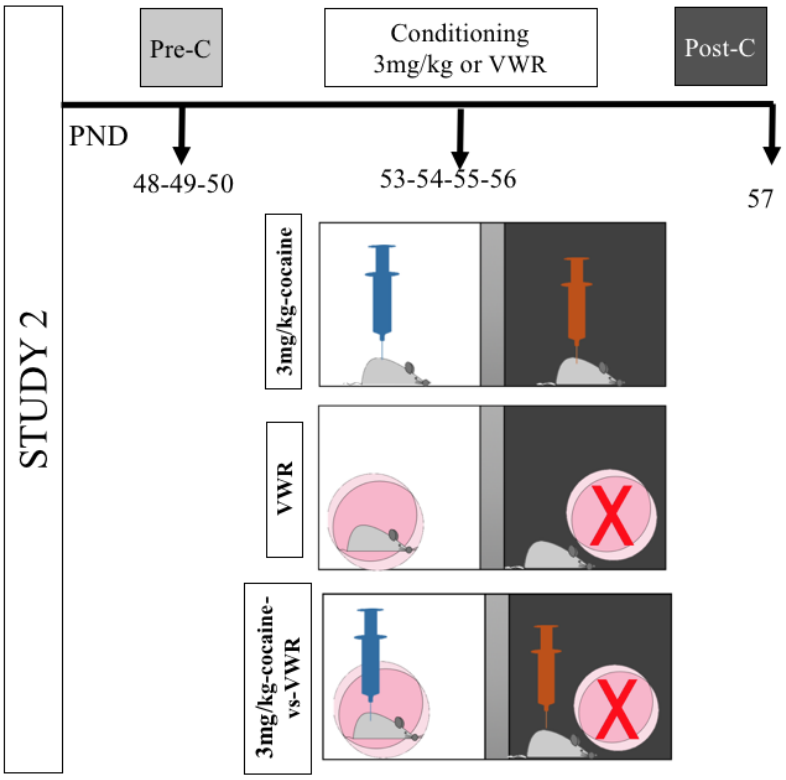



**Figure 2. Experimental design and experimental groups of Study 2**

### 2.4. Apparatus and procedures

**Voluntary running wheels**. Inside the home cages and for CPP induction, we employed traditionally-mounted vertical running wheels (15 cm diameter) with a solid surface. For intermittent exercise, procedure low profile running wheels (Med Associates Inc) were employed.

**Procedure of repeated social defeat (RSD).** The social defeat protocol was carried out following the procedure previously validated and described in detail by Ferrer-Perez and coworkers (2018, 2019). Briefly, each experimental animal under the RSD condition experienced 4 episodes of social defeat. Each episode began by introducing the "intruder" experimental animal into the home cage of the "resident" (the aggressive opponent) protected by a wire mess wall for 10 minutes. During this initial phase, the experimental animal was protected from direct attack, exposed to social interaction and species-typical threats from the male aggressive resident. Then, the wire mesh was removed from the cage to allow confrontation between the two animals for a 5-minute period. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 minutes. 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 (Rodríguez-Arias et al., 1998). In order to minimize the physical wounding during social defeats, the 5-minute direct encounters were interrupted if the intruder displayed a submissive supine posture for more than 8 seconds or if it was bitten by the aggressor more than 12 times (Burke et al., 2016).

**Conditioned Place Preference (CPP).** Place conditioning consisted of three phases and took place during the dark cycle following an unbiased procedure in initial spontaneous preference terms. For place conditioning, sixteen identical Plexiglas boxes with black and white equally sized compartments (30.7 × 31.5 × 34.5 cm) separated by a gray central area (13.8 × 31.5 × 34.5 cm) were used. In brief, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-minute period was recorded. Mice showing a strong unconditioned aversion (less than 33% of the time spent in both compartments) or preference (more than 67%) for any compartment were excluded from the study.

In the second phase (conditioning), animals underwent two pairings per day: subjects received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 min and, after an interval of 4 h, received cocaine immediately before being confined to the drug-paired compartment for 30 min. All animals in the Study 1 were conditioned with a dose of 1 mg/kg of cocaine while a group of 15 animals from Study 2 followed the same protocol but with a higher dose of cocaine (3mg/kg).

Additionally, in Study 2, two experimental groups underwent a variation of this procedure. Following a similar protocol, one group (VWR, n=21) was confined to one compartment with a free running wheel for 30 min and, after an interval of 4h, was confined to the other compartment with a locked wheel. The remaining group of animals (3mg/kg-cocaine-vs-VWR, n=17) experienced a conditioning procedure with the pairing of an active wheel and an injection of saline in one compartment and a dose of 3mg/kg cocaine and a locked wheel on the other compartment.

In the third phase (post-conditioning; Post-C), the time spent by mice in each compartment during the 15-minute observation period was recorded. The difference in

seconds between the time spent in the drug-paired or wheel-paired compartment in the Post-C test and that spent in the Pre-C test is a measure of the degree of conditioning induced. If this difference is positive, then the drug administration or the activity in the wheel is considered to have induced a preference for the paired compartment, whereas the opposite indicates the development of aversion. Additionally, in Study 2, the conditioning score was calculated for each mouse based on the difference between the time spent in the drug-paired or wheel paired compartment during the Post-C and Pre-C tests.

All groups from Study 1 for which a preference for the drug-paired compartment was established underwent an extinction session every 72 hours, which consisted of placing the mice in the apparatus for 15 minutes. This was repeated until the time spent in the drug-paired compartment by each group was similar to that of the Pre-C. Then the effects of non-contingent administration of a priming dose of cocaine were evaluated 24 hours after the confirmation of extinction. Reinstatement tests were the same as those for the Post-C (free ambulation for 15 minutes), except for the fact that mice were tested 15 minutes after administration of 0.5mg/kg of cocaine (half of the dose used for conditioning). This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

**Collection of biological samples**

Blood sampling for corticosterone determination was performed following the same procedure previously published in Montagud-Romero and coworkers (2017) and collected 30 min after the fourth episode of social defeat. Using the tail-nick procedure, the animal was immobilized and wrapped in a cloth so as to decrease experimental stress, and through a 2-mm incision at the end of the tail artery, 50 µl of blood was collected. Blood samples were kept on ice and plasma was separated from whole blood by centrifugation (5 minutes, 5000G) and transferred to sterile microcentrifuge tubes and stored at -80ºC until corticosterone determination.

Brain tissue sampling and plasma for IL-6 and BDNF determination was carried out 24 hours after finishing the CPP procedure in Study 1. Animals were sacrificed by cervical dislocation and then decapitated to collect blood from the neck in tubes coated with heparin. Blood samples were kept on ice and plasma was separated from whole blood

by centrifugation (5 minutes, 5000G) and transferred to sterile 0.2 ml microcentrifuge tubes. To obtain striatal and hippocampal tissue samples, brains were removed immediately after decapitation and dissected following the procedure described by Heffner and coworkers (1980). Plasma and tissue samples were stored at -80ºC until IL-6 and BDNF determinations.

**Determination of corticosterone, IL-6 and BDNF levels.** For the determination of plasmatic corticosterone levels, samples were diluted with the reagents provided in the EIA kit obtained from Enzo Life (Ref: ADI-900-097) and analyzed following the manufacturer's instructions.

For brain BDNF and IL-6 determinations, tissues were first homogenized and prepared following the procedure previously described in detail in Ferrer-Perez and coworkers (2018). For striatal, hippocampal and plasmatic IL-6 concentration, we used a Mouse IL-6 ELISA Kit obtained from Abcam (Ref: ab100712) and for BDNF quantification, we used the Emax ImmunoAssay System obtained from Promega (Ref: G7610) that were performed according to the manufacturer's instructions. Final tissue concentrations were normalized to each sample protein levels determined by the Bradford assay from ThermoFisher (Ref: 23227).

ELISA test results were read using an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software and final results were expressed in pg/mg for brain tissue samples and in pg/ml for plasma.

### 2.5. Statistical analyses

The establishment of CPP induced by cocaine in Study 1 was determined using a three-way ANOVA with two between subjects variables –Stress, with two levels (RSD and EXP), and -Activity, with 4 levels (SED, 1x4, 4x4, INT) - and a within-subjects variable—Days, with two levels (Pre-C and Post-C). In the groups showing CPP, extinction and reinstatement values were analyzed by a Student's t-test. A two-way ANOVA with the same two between-subjects variables (Stress and Activity) was employed to analyze the data of IL-6, and BDNF levels.

To analyze the CPP in Study 2, a two-way ANOVA with one between subjects variable – CPP Modality, with three levels (VWR, 3mg/kg-cocaine and 3mg/kg-cocaine-vs-

VWR) – and a within-subjects variable—Days, with two levels (Pre-C and Post-C)—was employed.

Data are presented as mean ± SEM and a p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS v24. In all cases, post-hoc comparisons were performed with Bonferroni tests.

## 3. Results

### 3.1. Physical activity modulates the increase in plasmatic corticosterone levels after an episode of social defeat

Data of the corticosterone concentration after the fourth social defeat episode is depicted in Figure 3. The ANOVA showed a significant effect of the variable Activity [$F(3,57) = 3.629$, $p = 0.018$], Stress [$F(3,57) = 6.899$, $p = 0.011$], and the interactions Activity x Stress [$F(3,57) = 6.806$, $p = 0.001$]. Post hoc analysis showed that animals housed in the sedentary housing condition (SED-RSD) and with one activity wheel (1x4-RSD) had higher corticosterone levels 30 min after the fourth episode of social defeat when compared to the levels obtained in non-stressed animals under the same housing condition (SED-EXP, $p<0.001$; and 1x4-EXP, $p<0.05$). The analysis also showed that, among the stressed animals, significant lower levels of corticosterone were recorded in the groups of animals housed with four activity wheels (4x4-RSD, $p <0.001$) and with a regimen of intermittent exercise (INT-RSD, $p <0.001$) when compared to RSD animals under sedentary housing conditions (SED-RSD). No differences were obtained in corticosterone levels of EXP animals in the different activity housing conditions.

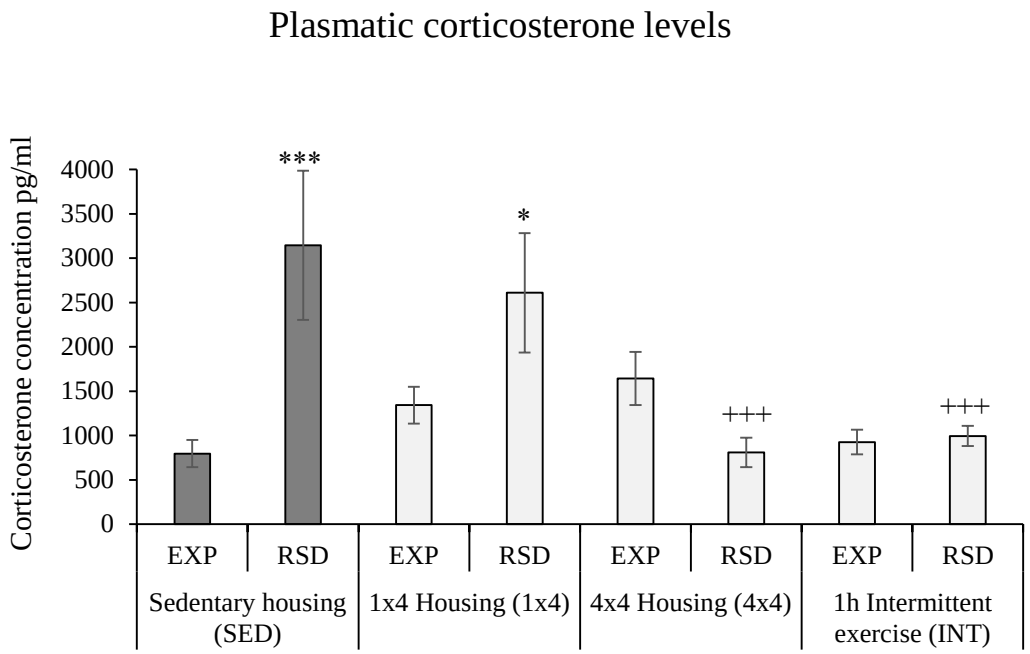



**Figure 3. Physical activity in the form of voluntary wheel running modulates the increase in plasmatic corticosterone levels after an episode of social defeat.** Animals were randomly assigned to the following groups according to physical activity condition: Sedentary housing (SED-EXP n=9; SED-RSD n=9); One activity wheel per cage (1x4-EXP n=8; 1x4-RSD n=8); Four activity wheels per cage (4x4-EXP n=8; 4x4-RSD n=8); Regimen of intermittent exercise (INT-EXP n=7; INT-RSD n=8). Data are presented as mean values corticosterone (pg/ml in plasma) ± SEM. Bonferroni's test *<0.05; ***p<0.001 significant difference in corticosterone levels between EXP vs. RSD within the corresponding group. +++p<0.001 significant difference in corticosterone levels vs. SED-RSD.

### 3.2. Increased physical activity enhanced cocaine conditioned rewarding effects

The ANOVA of the CPP data (Figure 4) showed a significant effect of the variable Days [$F(1,84) = 64.5$, $p < 0.001$] and the interactions Days x Activity [$F(3,84) = 8.679$, $p < 0.001$]. As expected, socially defeated animals under sedentary housing conditions (SED-RSD) developed CPP, since they spent more time in the drug-paired compartment in the Post-C than in the Pre-C test ($p=0.035$). This CPP was also registered in both exploration (EXP) and socially defeated animals (RSD), which were housed with one or four activity wheels per cage: 1x4-EXP $p= 0.003$; 1x4-RSD $p= 0.001$; 4x4-EXP $p<0.001$; 4X4-RSD $p<0.001$, and in EXP animals that followed the intermittent exercise regimen (INT-EXP $p= 0.031$). All groups that developed CPP were subjected to

extinction sessions. SED-RSD required 6 sessions, 1x4-EXP and 1x4-RSD required 4 sessions, 4x4-EXP required 10 sessions, 4x4-DSR, 5 sessions and INT-EXP, 12 sessions to extinguish the preference. Reinstatement of the preference after a priming dose of 0.5 mg/kg of cocaine was achieved in RSD groups (SED-RSD, 1x4-RSD and 4x4-RSD). No differences were observed in the time needed to extinguish this preference (2 sessions), and neither of these groups showed reinstatement after priming dose with 0.25 mg/kg cocaine.

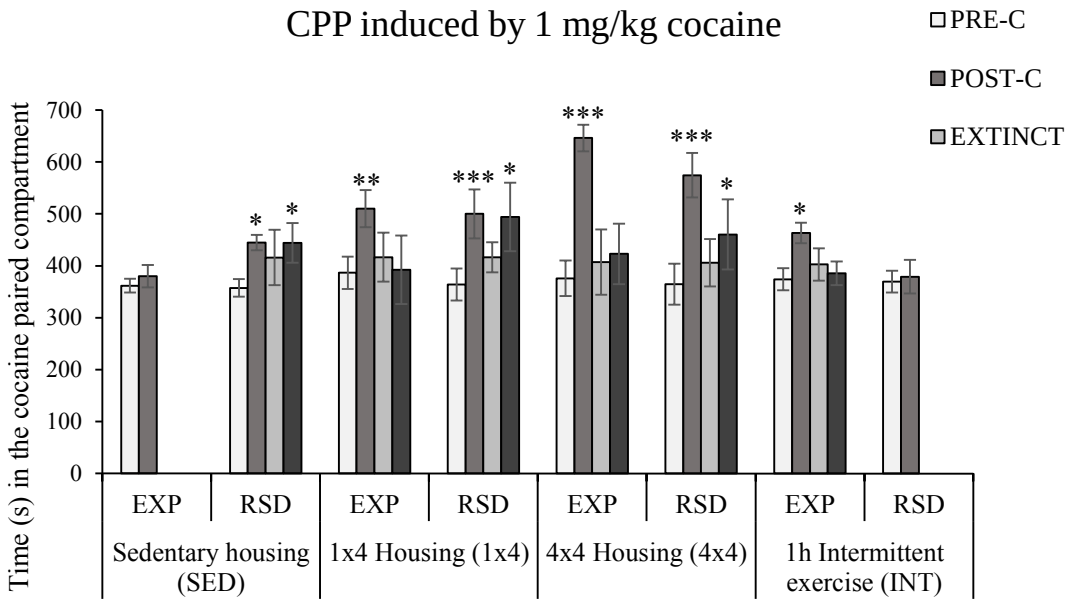



**Figure 4. Increased physical activity enhanced cocaine conditioned rewarding effects.** Animals were randomly assigned to the following groups according to their physical activity condition: Sedentary housing (SED-EXP n=12; SED-RSD n=12); One activity wheel per cage (1x4-EXP n=12; 1x4-RSD n=12); Four activity wheels per cage (4x4-EXP n=10; 4x4-RSD n=10); Regimen of intermittent exercise (INT-EXP n=12; INT-RSD n=12). The bars represent the time (s) spent in the drug-paired compartment before conditioning sessions in the PRE-C test (white bars), and after conditioning sessions in the POST-C test, in the last extinction session (EXTINCT, black bars), and in the reinstatement (REINST) test (dark gray bars). Data are presented as mean values ± SEM. Bonferroni's test *p<0.05; **p<0.01; ***p<0.001 significant difference in the time spent in the drug-paired compartment vs. PRE-C.

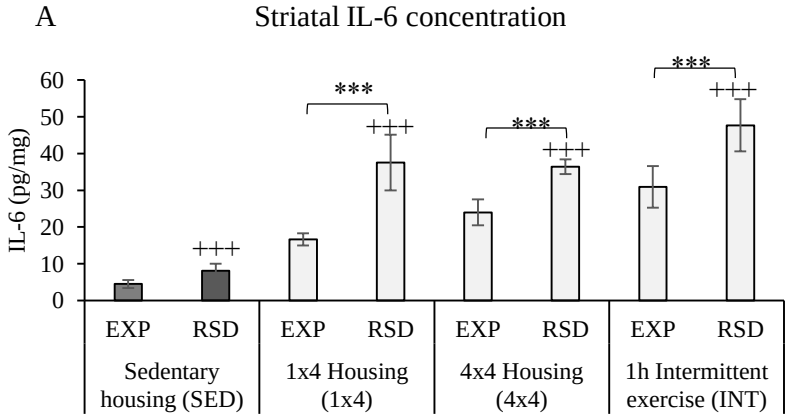
### 3.3. Physical activity in the form of wheel running increases IL-6 concentration in plasma, the striatum, and the hippocampus

The ANOVA of IL-6 levels in the striatum, the hippocampus and in plasma are represented in Figure 5. Results of IL-6 striatal levels (Figure 5A) showed a significant

effect of the variable Activity [F(3,48 = 17.988, p <0.001] and Stress [F(1,54) = 18.519 p <0.001]. Post-hoc analysis showed that animals under Sedentary Housing conditions (SED) have significantly lower levels of striatal IL-6 compared to animals exposed to VWR (p<0.001 in all cases). On the other hand, striatal IL-6 levels among socially stressed animals (SED-RSD, 1x4-RSD, 4x4-RSD and INT-RSD) were higher (p< 0.001) compared to their corresponding non-stressed groups (SED-EXP, 1x4-EXP, 4x4-EXP and INT-EXP).

Results of IL-6 levels in the hippocampus (Figure 5B) showed a significant effect of the variable Activity [F(3,48 = 9.606, p <0.001]. Post-hoc analysis showed that animals under Sedentary Housing conditions (SED) have significantly lower levels of striatal IL-6 compared to animals housed with one (1x4, p<0.001) or four (4x4, p<0.01) activity wheels, or those in intermittent exercise (INT, p<0.001).

Finally, the ANOVA for IL-6 levels in plasma (Figure 5C) showed a significant effect of the variable Activity [F(3,48 = 4.258 p = 0.01] and Stress [F(1,48) = 4.158, p =0.047]. Post-hoc analysis showed that animals housed with one activity wheel (1x4) had significantly higher levels of plasmatic IL-6 compared to animals under sedentary housing (SED, p<0.05) and under a regimen of intermittent activity (INT, p<0.05). As obtained in the striatum, plasmatic IL-6 levels among socially stressed animals (SED-RSD, 1x4-RSD, 4x4-RSD and INT-RSD) were higher (p<0.05) compared to that of the non-stressed groups (SED-EXP, 1x4-EXP, 4x4-EXP and INT-EXP).



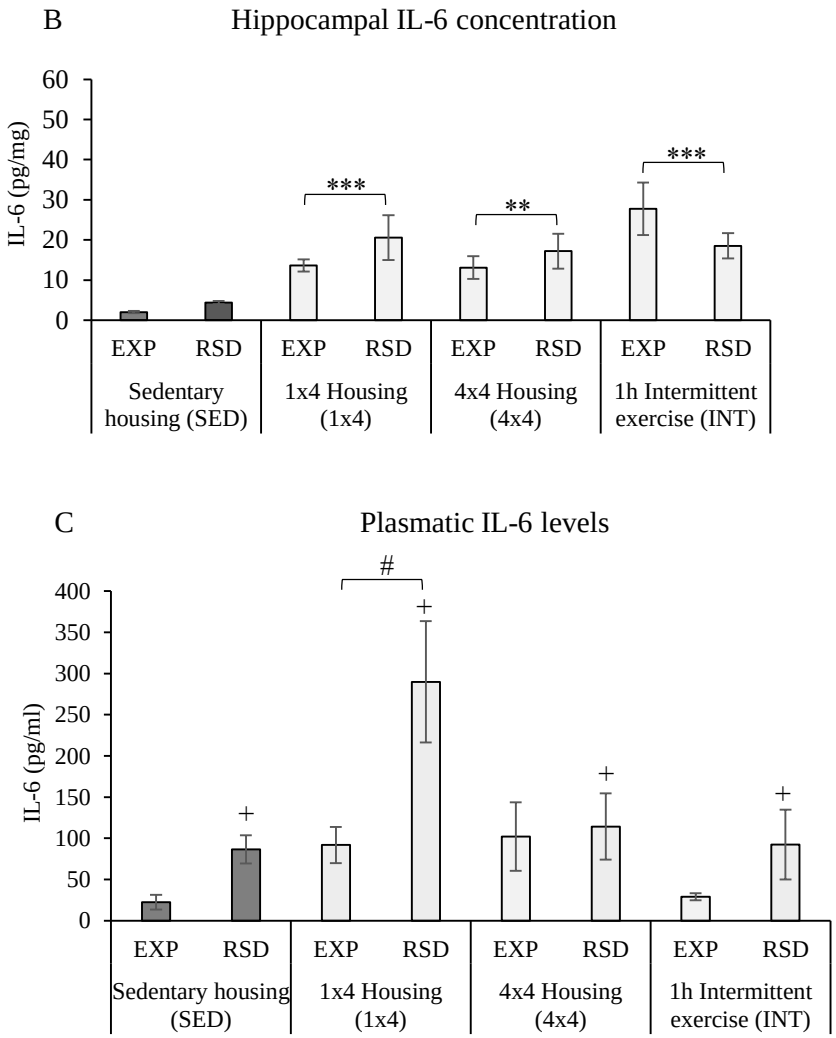



**Figure 5. Effects of voluntary wheel running on striatal, hippocampal and plasmatic levels of IL-6**. Sedentary housing (SED-EXP n=6; SED-RSD n=6); One activity wheel per cage (1x4-EXP n=8; 1x4-RSD n=8); Four activity wheels per cage (4x4-EXP n=8; 4x4-RSD n=7); Regimen of intermittent exercise (INT-EXP n=7; INT-RSD n=7). Data are presented as mean values of IL-6 (pg/mg protein in brain tissue and pg/ml in plasma) ± SEM. Bonferroni's test *<0.05; **p<0.01; ***p<0.001 significant difference in IL-6 levels vs. Sedentary housing (SED). +p<0.05 significant difference in IL-6 levels between EXP vs. RSD; #p<0.05 significant difference in IL-6 levels between 1x4 vs. SED and INT.

### 3.4. Physical activity in the form of wheel running increases BDNF concentration in the striatum and the hippocampus

The ANOVA performed for the BDNF concertation in the striatum (Figure 6A) showed an effect of the variable Activity [$F(3,46) = 19.302$, $p < 0.001$]. Animals housed with one (1x4, $p<0.001$) or four (4x4, $p<0.01$) activity wheels, or with a regimen of

intermittent activity (INT, p<0.001) had higher striatal BDNF levels than animals with sedentary activity (SED). Additionally, BDNF levels in animals housed with 4 activity wheels (4x4) were lower when compared to animals housed with one activity wheel (1x4, p<0.05) or with intermittent access (INT, p= 0.025).

The ANOVA performed for the BDNF concertation in the Hippocampus (Figure 6B) showed an effect of the variable Activity [F(3,46) = 129.513, p <0.001]. Similar to that obtained in the striatum, animals housed with one (1x4, p<0.001) or four (4x4, p<0.01) activity wheels, or with a regimen of intermittent exercise (INT, p<0.001) had higher BDNF levels in the hippocampus than animals with sedentary physical activity (SED). Additionally, these BDNF levels were higher in animals housed with one activity wheel (1x4) when compared with the levels of animals housed with four activity wheels (4x4, p<0.001) or with intermittent access (INT, p<0.001).

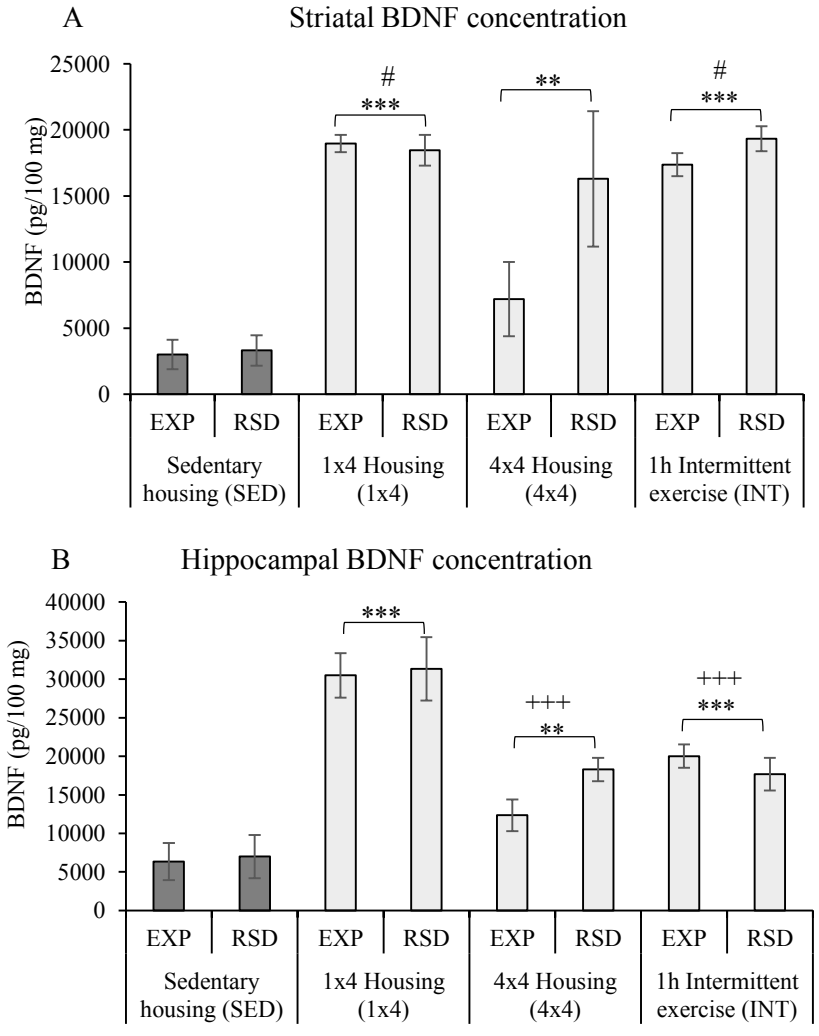


**Figure 6. Effects of voluntary wheel running on striatal and hippocampal BDNF levels**. Sedentary housing (SED-EXP n=5; SED-RSD n=7); One activity wheel per cage (1x4-EXP n=7; 1x4-RSD n=6); Four activity wheels per cage (4x4-EXP n=9; 4x4-RSD n=6); Regimen of intermittent exercise (INT-EXP n=7; INT-RSD n=7). Data are presented as mean values of BDNF (pg/100 mg protein) ± SEM. Bonferroni's test **p<0.01; ***p<0.001 significant difference in BDNF levels vs. Sedentary housing (SED). #p<0.05 significant difference in BDNF levels vs. 4x4; +++p<0.001 significant difference in BDNF levels vs.1x4.

### 3.5. Voluntary wheel running induced CPP and neutralized the development of preference for an effective dose of cocaine (3mg/kg)

The ANOVA performed for the CPP data of the second study CPP data (see Figure 7A) showed a significant effect of the factor Days [$F(1,50) = 13.533$, $p < 0.001$] and the interaction Days x CPPModality [$F(2,50) = 3.571$, $p = 0.036$]. Post-hoc analysis showed that animals conditioned with a voluntary running wheel associated with one compartment and a locked wheel in the other compartment spent a significantly longer time in the active wheel compartment in the Post-C than in the Pre-C ($p<0.01$). Similarly, animals that underwent a CPP protocol induced by 3 mg/kg, which were controlled with an injection of saline in the non-drug paired compartment, developed CPP for the drug paired compartment ($p<0.001$). Moreover, the time spent in the drug-paired compartment was significantly longer when compared to the time spent in the drug-paired compartment by animals also conditioned with 3mg/kg of cocaine but who had the non-drug paired compartment controlled by an injection of saline and with access to an active running wheel ($p<0.05$). This difference is also manifest considering the conditioning score (see Figure 7B), ANOVA revealed an effect of the variable CPPModality [$F(2,50) = 3.571$; $p = 0.036$]. Mice conditioned with 3mg/kg of cocaine and controlled with saline had higher conditioning scores than those also conditioned with 3mg/kg of cocaine but with an active wheel and saline paired with the other compartment ($p<0.05$).

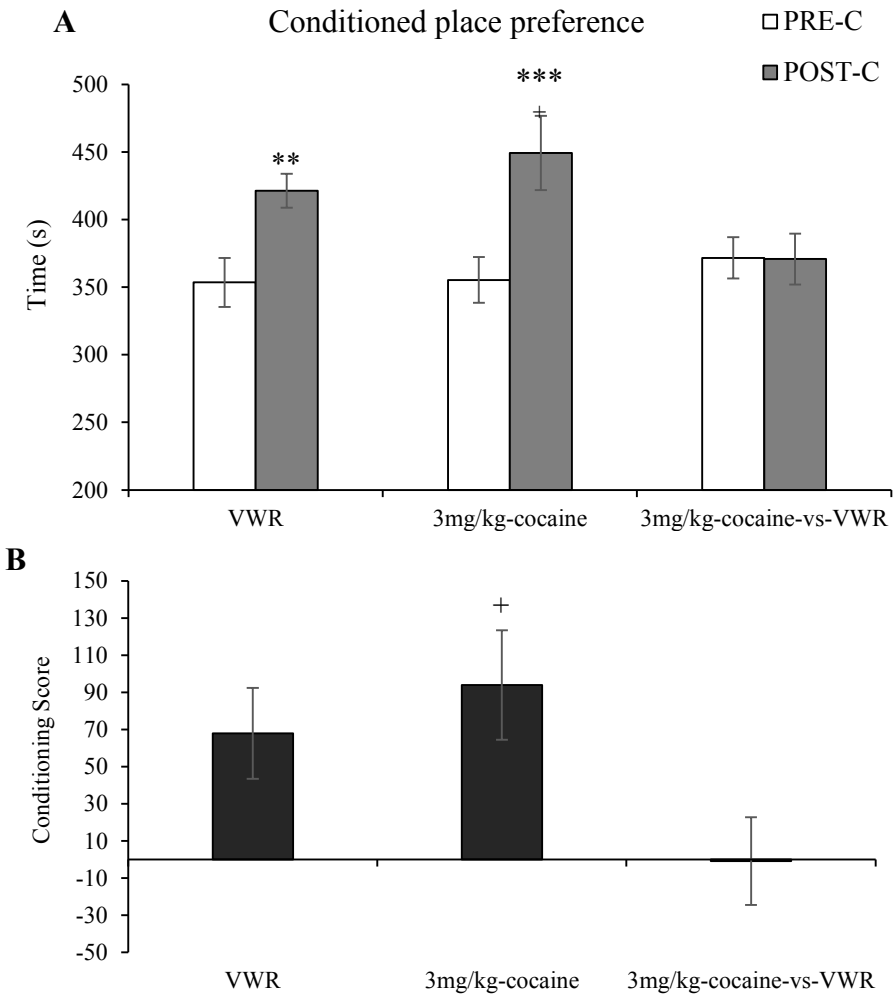



**Figure 7. Voluntary wheel running induced CPP and neutralized the development of a preference for an effective dose of cocaine (3mg/kg).** (A) The bars represent the time (s) spent in the active wheel paired compartment (VWR group) or in the cocaine drug-paired compartment (3mg/kg-cocaine and 3mg/kg-cocaine-vs-VWR groups). White bars represent the time (s) spent during the PRE-C test and dark grey bars, after conditioning sessions in the POST-C test. **p<0.01; ***p<0.001 significant difference in the time spent in the paired compartment vs. PRE-C. + p< 0.05 differences in the time spent during the Post-C between the groups of 3mg/kg-cocaine and 3mg/kg-cocaine-vs-VWR. (B) Bars represent the conditioning score (CS), calculated as the time spent in the wheel-paired compartment (VWR group) and in the drug-paired compartment (3mg/kg-cocaine and 3mg/kg-cocaine-vs-VWR groups) in the Pre-C test minus the time spent there in the Post-C test. + p<0.05 significant between the groups 3mg/kg-cocaine and 3mg/kg-cocaine-vs-VWR.

## 4. Discussion

Exercise in the form of VWR promoted a decrease in the endocrine response to social stressors. However, despite these effects, it did not show a buffering effect on the stress-induced increase in the conditioned rewarding effects of cocaine. We found that animals with access to running wheels, with the exception of the INT-RSD group, developed CPP induced by a subthreshold dose of cocaine (1mg/kg), a dose that is not effective in animals under homeostatic conditions (Montagud-Romero et al., 2018). In light of previous reports, we only expected to observe CPP in socially defeated mice, as social stress has been proven to sensitize the reward system by increasing cocaine conditioned rewarding effects (Montagud-Romero et al., 2018; Ferrer-Pérez et al., 2018, 2019). A common mechanism that could explain this enhanced response to cocaine is that increased physical activity in the three regimen studied—intermittent access to one activity wheel (INT) or continuous access to one (1x4) four (4x4) activity weels—promoted an elevation of striatal and hippocampal levels of the pro-inflammatory cytokine IL-6, effect that was exacerbated in animals that also experienced social defeat. Above, we have mentioned that increases of IL-6 can sustain the enhanced response to cocaine in CPP (Ferrer-Pérez et al., 2018). Another possible factor that could explain this increased CPP is the fact that physical exercise enhanced mice BDNF concentrations in the hippocampus and the striatum. Enhanced levels of this neurotrophin in the mentioned structures have been previously linked to enhanced learning performances in associative learning tasks, such as CPP. However, these mechanisms do not explain the protective effect observed only in the INT-RSD group.

Animals housed in sedentary condition presented a significant increase of plasmatic corticosterone levels measured 30 minutes after the last agonistic encounter when compared with the hormone levels of animals that had undergone a similar experimental manipulation but without experiencing social defeat. This rise in cortisol levels was not observed in socially defeated animals housed with four activity wheels (4x4-RSD) or with intermittent access to VWR (INT-RSD). These results are in agreement with previous studies that also showed that regular physical exercise blunts the neuroendocrine response to stressors and promotes a decrease in corticosterone response to acute stress challenges (Lynch et al., 2019; Sasse et al., 2008). This effect can be mainly explained by the capacity of physical exercise to act as an acute stressor (Hayes

et al., 2008; Lynch et al., 2019), which, after repeated exposures, promotes an adaptation of the hypothalamic pituitary adrenal axis, thereby decreasing reactivity to further challenges (Droste et al., 2003; Pietrelli et al., 2018; Sasse et al., 2008). However, we did not record this protective effect in the groups of animals housed with one activity wheel (1x4-RSD). We think that, as animals had only one activity wheel per four mice, there was a competition, so to speak, for that resource that also causes stress. In fact, other researchers have previously described that limited environmental enrichment items can promote aggressive competition (Howerton et al., 2008; Nevison et al., 1999). Additionally, this aggressiveness may be highlighted with the fact that animals being denied access to the resource can also display increased aggression as an indicator of exercise withdrawal (Hoffmann et al., 1987).

There is a vast body of scientific publications that confirm the inflammatory effects of social defeat stress, and its potential to induce a sensitization of the immune system to further inflammatory challenges (Ambrée et al., 2018; Chan et al., 2019; Ferrer-Pérez et al., 2018). Moreover, the susceptibility of an individual to display inflammatory states after stress experiences has been directly related to the susceptibility to develop stress-associated pathologies (see revision in Cathomas et al., 2019). Given its promising effects in blocking the endocrine response to stressors, we expected that VWR would also have a buffering effect against the sensitization of the immune system induced by social stress. However, our results point in the opposite direction, and animals in the conditions of increased exercise (1x4, 4x4 and INT) had higher IL-6 levels in the striatum, hippocampus and plasma than animals housed in the sedentary condition, levels which were even higher if they also experienced social defeat stress. While some studies and revisions have highlighted the anti-inflammatory effects of exercise in both humans and rodents (Flynn et al., 2007; Gleeson et al., 2011), these beneficial effects seem to be dependent on the exercise intensity, duration as well as the initial inflammatory state (Eldomiaty et al., 2017; Eyre & Baune, 2012; Pedersen & Hoffman-Goetz, 2000; Pinto et al., 2018).

During physical effort, there is a peak of release of IL-6 by muscles that rapidly decrease after exercise (Febbraio & Pedersen, 2002; Pedersen et al., 2001, 2011; Pinto et al., 2018) and its function has been connected with muscle metabolism processes (Pedersen & Febbraio, 2008). This acute pro-inflammatory signaling from exercise is

followed by a transient anti-inflammatory state caused by the induction of anti-inflammatory cytokines such as IL-10 and by the inhibition of the synthesis of other pro-inflammatory cytokines such as TNF-α (Gleeson & Walsh 2012; Petersen & Pedersen, 2005). This anti-inflammatory post-effect of exercise has been proven to be effective to regulate inflammatory patterns in states with elevated chronic inflammation such as in depression, type 2 diabetes or obesity (Beavers et al., 2010; Eldomiaty et al., 2017; Peppler et al., 2016; Petersen & Pedersen, 2005; Pinto et al., 2012; Rocha-Rodrigues et al., 2017). In subjects with homeostatic levels of inflammatory markers, it seems that the volume of exercise and the intensity determines whether it exerts an anti-inflammatory or an inflammatory effect (Balducci et al., 2010). Increases in intensity and volume are translated into increases in IL-6 plasmatic concentrations, which can modify the net result of this response, turning an initial anti-inflammatory effect into an inflammatory effect (Lombardi et al., 2019). In an interesting study with humans, Paolucci and collaborators (2018) found that, while moderate exercise reduced TNF-α and improved mood, high intensity exercise promoted higher levels of perceived stress and inflammation.

In this regard, we think that the enhancement of central and peripheral IL-6 levels registered in animals with access to voluntary running wheels is caused by a pattern of excessive exercise. In fact, some researchers claim that wheel running behavior in mice shares some characteristics of a stereotypical or addictive behavior, which also can turn into maladaptive and obsessive behaviour (Novak et al., 2012). For instance, in a regimen of limited hours of access to food, animals will choose to run on the wheel rather than spend the time eating (Aoki et al., 2017). In a previous research study, we found that increased IL-6 signaling can predict stress susceptibility to develop a sensitization to the rewarding effects of cocaine, while the prevention of this inflammatory state reversed the stress-enhancement of cocaine CPP (Ferrer-Pérez et al., 2018). Thus, the enhanced inflammatory state induced by intense VWR, together with the inflammatory effect of social defeat, can be used to explain CPP development in our experimental mice.

Another factor that can also account for the enhanced CPP observed in non-stressed groups is the fact that physical activity in running wheels has been liked to increase levels of BDNF (Baj et al., 2012; Smith et al., 2008; Vaynman et al., 2004). We

detected enhanced BDNF levels in the striatum and the hippocampus of animals with physical activity, with the hippocampal levels being even higher in animals housed with one activity wheel (1x4). BDNF sustains plastic process in the brain, as well as neurogenesis (Liu & Nusslock, 2018) and enhanced levels of this neurotrophin, mainly in the hippocampus, have been linked to enhanced cognitive performance with enhanced memory consolidation and learning (Erickson et al., 2011). As CPP establishment is sustained by an associative learning process between the interoceptive response and environmental cues (Baruch et al., 2004), an enhanced capacity of learning and memory would impact CPP outcomes (Smith et al., 2008). In fact, some researchers have previously reported that experiences of voluntary exercise increased BDNF in the hippocampus and promoted grater magnitudes of CPP induced by morphine (Eisenstein & Holmes, 2007) and cocaine (Smith et al., 2008). Although these results suggest that VWR increases sensitivity to the conditioned rewarding effects of cocaine, this should not mean that physical exercise increases susceptibility to cocaine addiction. In fact, physical exercise can also promote positive outcomes such as increased learning, which may also accelerate the extinction of context-associated drug memories, stimulating the creation of new memories of the non-availability of drug reward.

Previous results lead us to wonder about the intrinsic rewarding capacity of VWR. Therefore, in the second study, we confirmed that VWR has a rewarding effect that is able to counteract the cocaine potential to establish CPP. First, we confirmed, following the same conditioning procedure used for cocaine, that VWR has enough rewarding properties to promote place preference for the wheel-paired compartment in naïve animals without previous experiences with activity wheels. This result was not surprising, as other researchers have reported that rats and mice learn to lever-press for access to a running wheel (Belke & Garland, 2007) and develop CPP to the after-effects of running episodes (Lett et al., 2000, 2001). The interesting result is that these rewarding properties are strong enough to compete against the conditioned rewarding properties of an effective dose of cocaine (3mg/kg). When this dose was associated with one compartment and controlled with an injection of saline in the other, cocaine induced a strong preference for the drug-paired compartment, phenomenon that has also been reported in previous studies (Reguilón et al., 2017). However, when one compartment was associated with 3mg/kg of cocaine effects and the other with a VWR experience, animals did not develop conditioned preference for any compartment. As we previously

discussed, physical exercise is considered an alternative reinforcer, but the net effect of physical exercise intervention, whether it can exert a protective effect acting as a hedonic substitute or enhance drug reward, seems to be related with the moment in which the intervention is applied.

Our results endorse that voluntary wheel running activates the same brain circuits as other natural rewards and drugs of abuse (Brené et al., 2007; Olsen, 2011). Access to reinforcers cause neuroplasticity in the mesolimbic dopaminergic system, which promotes the maintenance of rewarding behaviors (Koob & Volkow, 2010). Akin to our results, Lynch and coworkers (2019) found that VWR, rather than having a protective effect, increased ethanol preference. They posited that this effect was caused by the fact that, if physical exercise is experienced before contact with the drug, it sensitizes and prepares the reward system to the rewarding effects of ethanol, which act as a hedonic substitute of wheel running. However, when the first experience with VWR is concurrent with the drug, it exerts a protective effect, as it competes with drug potential to establish context memories, as we found in Study 2. Other studies have also reported protective effects of concurrent access to wheel running during drug memory acquisition, such as suppression of cocaine self-administration (Cosgrove et al., 2002) and decreased cocaine-seeking behavior during extinction (Zlebnik et al., 2010).

Finally, it seems that the moment when wheel running can exert its maximum benefit is after the acquisition of drug memories. For instance, Solinas and coworkers (2008) showed that, after cocaine CPP establishment, animals housed in an enriched environment with free access to activity wheels were protected against cocaine-primed reinstatement of the conditioned preference. Similarly, Sikora and coworkers (2018) found that animals housed with access to activity wheels after methamphetamine, nicotine and heroine acquisition of self-administration behavior reduced drug-seeking during abstinence.

To sum up, our results showed that VWR increased mice sensitivity to the conditioned rewarding effects of cocaine. This result is relevant because contextual cues associated with the drug play a critical role in the development and maintenance of addictive disorders and can also promote relapse. This increase in vulnerability to develop cocaine-induced CPP can be promoted by the inflammatory potential of physical exercise and its improvement over associative learning through BDNF actions in the

hippocampus and the striatum. Additionally, this effect may also be caused by a direct sensitization of the reward pathway by VWR, which in Study 2 proved to be a powerful natural reinforcer. Together, these results highlight that physical exercise deeply impacts the organism's response to stress and drugs. Thus, we believe that a good design of a physical intervention should take into consideration factors such as duration and moment of the exercise in order to be an effective tool in the management of stress and abstinence. This research work would help to optimize the designs of physical interventions as it increases the understanding about the neurobiological effects of physical exercise.

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# Study 7

Characterization of the neurovascular and immune consequences of chronic social defeat stress and its role in stress-induced depression-like behavior

Ferrer-Pérez, C.

*Pilot Study*

## Characterization of the neurovascular and immune consequences of chronic social defeat stress and its role in stress-induced depression-like behavior

### 1. Introduction

This final study was carried out during my research stay in the Russo Laboratory at the Icahn School of Medicine at Mount Sinai. In the following lines, I will present the main findings of this pilot study structured as a scientific journal article.

There is a vast number of scientific publications, from both pre-clinical and human studies, showing that social stress can induce deep alterations in the immune system (revision in Dantzer, 2017). In turn, immune system dysfunctions that lead to inflammatory states have been linked to an increased risk of stress-induced disorders, especially mood disorders (Slavich & Irwin, 2014). Moreover, the susceptibility of the immune system to be shifted into a inflammatory state after social stress experiences has been connected to the susceptibility of displaying stress-induced psychological and psychological impairments (Hodes et al., 2014). However, the exact mechanism of interaction of the peripheral immune system and the central immune system that promotes the neuro-inflammatory response within the central nervous system (CNS) is not yet completely understood. The blood brain barrier (BBB) under basal conditions restricts the communication between the CNS and the systemic circulatory system, preventing the arrival of cells and cytokines from the periphery to the CNS (Engelhardt et al., 2017). Notwithstanding, during stressful situations, the permeability of the BBB can be compromised, allowing peripheral cells and cytokines to enter the brain (Ménard et al., 2017; Rodríguez-Arias, 2015).

In the present study, we evaluated the impact of chronic social defeat stress (CSDS) on the integrity of the BBB by quantifying the expression of the tight junction protein claudin 5 (cldn5). We also aimed to define its role in the appearance of stress-induced depressive-like behavior. Cldn5 is a major adhesion molecule of endothelial cells. Decreases of this molecule has been linked to a more permeable BBB (Günzel & Yu, 2013). Additionally, we performed immunohistochemical image studies in order to determine the infiltration of peripheral immune cells into brain parenchyma.

We postulate that there is a link between the level of disturbance in the permeability of the BBB after stress and the level of suffering pathological stress consequences. We expect that a higher increase in the leakiness of the BBB after stress will predict an increased susceptibility to the depressogenic effects of social stress.

The present pilot study is part of an ambitious project that aims to carry out a complete characterization of the immune response trigged by social defeat stress and the neuro-immune interaction that leads to an increased susceptibility to suffering from negative stress consequences.

## 2. Material and Methods

### 2.1. Animals

A total number of 60 *ccr2*$^{RFP}$:: *cx3cr1*$^{GFP}$ mice were bred at the School of Medicine in Mount Sinai housing facilities. At 8–10 weeks of age, they were rehoused following CSDS protocol. In addition to the experimental animals, 50 retired male CD-1 breeders were acquired from Charles River Laboratories and used as aggressors.

Animals were maintained in a 12-h/12-h light/dark light cycle with lights on at 07:00. Food and water was available ad libitum. Procedures involving mice and their care were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the ISMMS Animal Care and Use Committee.

### 2.2. Experimental design

The experimental procedure is depicted in Figure 1. *Ccr2RFP:: cx3cr1GFP* mice were divided into two stress conditions: non-stressed control (CON) and animals exposed to the CSDS protocol (explained below).

A day after finishing the CSDS procedure, 18 experimental mice were discarded, as they presented elevated wounding scores caused during the CSDS. The rest of the animals were tested for social interaction (SI) behavior. Using the measures scored in this test, animals were classified as stress-susceptible (SUS) and stress-resilient (RES). Then, 48 h after finishing the CSDS procedure, animals were sacrificed and brain tissue

samples were collected. A total of 40 brain samples were employed for the qPCR analysis while 12 brains were sliced and used for immunohistochemistry.

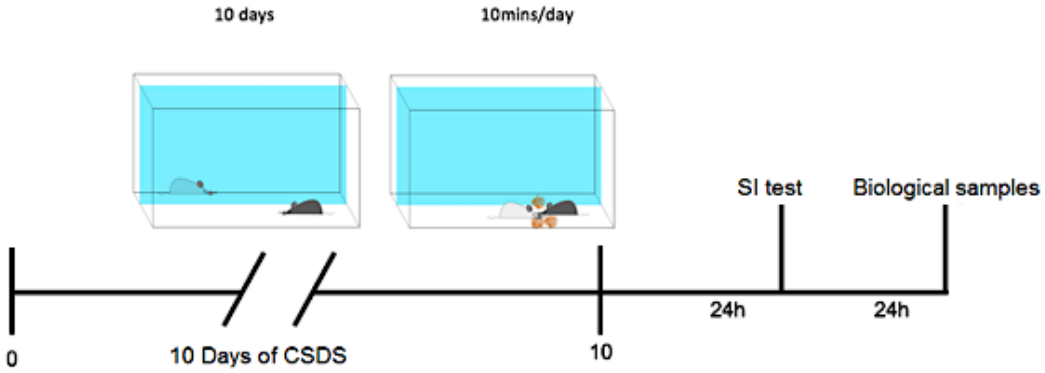


**Figure 1. Experimental design**

### 2.3. Apparatus and procedures

#### Chronic social defeat stress (CSDS).

The CSDS protocol was performed as previously described in detail by Golden and collaborators (2011). Five days prior to the initiation of the CDS protocol, aggressive CD-1 mice were screened to confirm appropriate levels of aggressive behavior. Any CD-1 mouse showing a latency to attack of over 3 minutes was withdrawn from the experiment. Then, aggressive mice were housed in social defeat cages (26.7 cm width × 48.3 cm depth × 15.2 cm height) on one side of a clear perforated Plexiglas divider (0.6 cm × 45.7 cm × 15.2 cm) 24 h prior to the beginning of the CSDS protocol. Then, experimental *ccr2*$^{RFP}$:: *cx3cr1*$^{GFP}$ mice were also housed in the cage on the other side of the Plexiglas divider. Each day, experimental mice were exposed to physical defeat with a CD-1 for 10 min once per day over 10 consecutive days. After each defeat, the experimental mice were placed on the opposite side of the divider, allowing sensory contact over the subsequent 24-h period. Control (CON) mice were housed two per cage in each side of a perforated divider, following an analogue protocol of rotation with the exception of mice not experiencing social defeat. After the last social defeat, animals were then individually housed and the SI test was conducted 24 h later.

**Social interaction (SI) test.**

The SI protocol was performed as previously described in detail by Golden and collaborators (2011). The procedure was recorded using a tracking system (Ethovision 11.0, Noldus Information Technology). Under dim illumination conditions, experimental animals were placed in a white open field arena (42 cm × 42 cm × 42 cm) with a small wire animal cage placed at one end. During a 150-s period, the baseline exploratory behavior in absence of the social target was recorded. Lastly, the exploratory behavior was recorded again for 150 s, but this time with the presence of a novel social target mouse inside the small wire cage. The mean of time spent in the interaction zone (closer to the wire animal cage) and in the corner zones was estimated. The SI ratio was obtained by dividing the time spent in the interaction zone when the target mice were present divided by the time spent in the interaction zone during the baseline measure. Experimental mice with an SI ratio below 1.0 were considered stress-susceptible (SS), while mice with an SI ratio above 1.0 were considered stress-resilient (RES).

**Cldn5 mRNA quantification.**

A day after the SI test, animals were sacrificed by rapid decapitation and bilateral punches (14-gauge) of the nucleus accumbens were collected from 1-mm coronal slices, then immediately stored at -80°C degrees for qPCR analysis. Samples were analyzed following the exact procedure carried out by Ménard and collaborators (2017). Briefly, for RNA, extraction samples were homogenized using TRIzol for and then treated with chloroform for separation. Then, a RNeasy Kit (Qiagen) was used to treat the samples following the manufacturer's instructions. Then, the RNA quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA was reverse transcribed to cDNA with qScript (Quanta Biosciences). Following this, 3 µl of cDNA of each sample was used for 40 cycles qPCR reaction using the Cldn5 primer NM_0138051. Data analysis was carried out using the ΔΔC(t) method. Measures were normalized to gapdh (mouse) housekeeping gene.

**Immunohistochemistry and imaging.**

Brains of perfused mice were rapidly frozen and stored at -80°C. A day before slicing, brains were transferred to a -20°C freezer. Using a cryostat, 12-µm-thick slices of the whole brain were obtained. Following this, they underwent a blocking incubation of 2 h in 5% donkey serum in PBS. Then, they were incubated overnight with the primary antibodies CD31 (anti-rat-CD31, 1:150), CD206 (goat) RFP (rabbit). After washing with PBS for three times, samples were incubated with secondary antibodies (anti-rat-Cy5, anti-rabbit-Cy3 and anti-goat-Alexa405) for 2 h. Slices were mounted, cover slipped and visualized using a LSM-780 confocal microscope (Carl Zeiss). Images were taken using a 20× lens with a resolution of 1056 × 288 and a 2.0 zoom. For co-localization, we used a 40× lens with oil immersion. Post-imaging analysis was carried out using the Image J (NIH) software for cell and area counting.

**2.4. Statistical analysis.**

Data of cldn5 mRNA expression was analyzed using a one-way ANOVA with the variable Group, with three levels (CON, RES and SUS). Bonferroni was used as a post-hoc test. Statistical significance was considered at $p<0.05$. Analyses and graphs were performed using GraphPad Prism software (GraphPad Software Inc.).

## 3. Results

**3.1. Chronic social defeat stress downregulated cldn5 mRNA expression**

ANOVA of qPCR data showed an effect of the variable Group [$F(1,37) = 9.976$; $p < 0.001$]. The post-hoc analysis showed that cldn5 mRNA was significantly reduced in the nucleus accumbens of all stressed animals, regardless of being RES ($p<0.01$) or SUS ($p<0.001$) when compared to cldn5 mRNA levels in control (CON) mice.

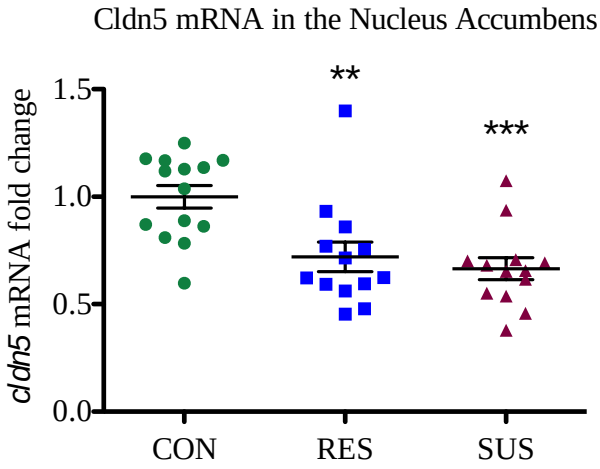


**Figure 2. qPCR data revealed a significant decrease in gene expression of cldn5 mRNA in chronically stressed mice when compared to non-stressed animals (CON).** After the CSDS protocol, animals were classified as stress-resilient (RES, n=13) or stress-susceptible (SUS, n=13) considering their SI test scores. Each dot represents data from a single subject and mean ± SEM for each group. Gene expression level is reported as fold change with normalization of mRNA expression to CON (n=14) mice. **p < 0.01; ***p < 0.001 vs. CON.

### 3.2. Characterization of peripheral immune cell infiltration into the brain after chronic social defeat stress

We measured the presence of peripheral immune monocytes (ccr2$^{RFP}$) in two brain structures: the nucleus accumbens and the choroid plexus. The quantification of the number of positive ccr2$^{RFP}$ cells did not show differences between non-stressed and stressed mice (Figure 3A and B). Posterior analysis by immunohistochemical imaging showed that infiltrated peripheral monocytes accumulated within the perivascular space but did not infiltrate into the brain parenchyma (Figure 3C).

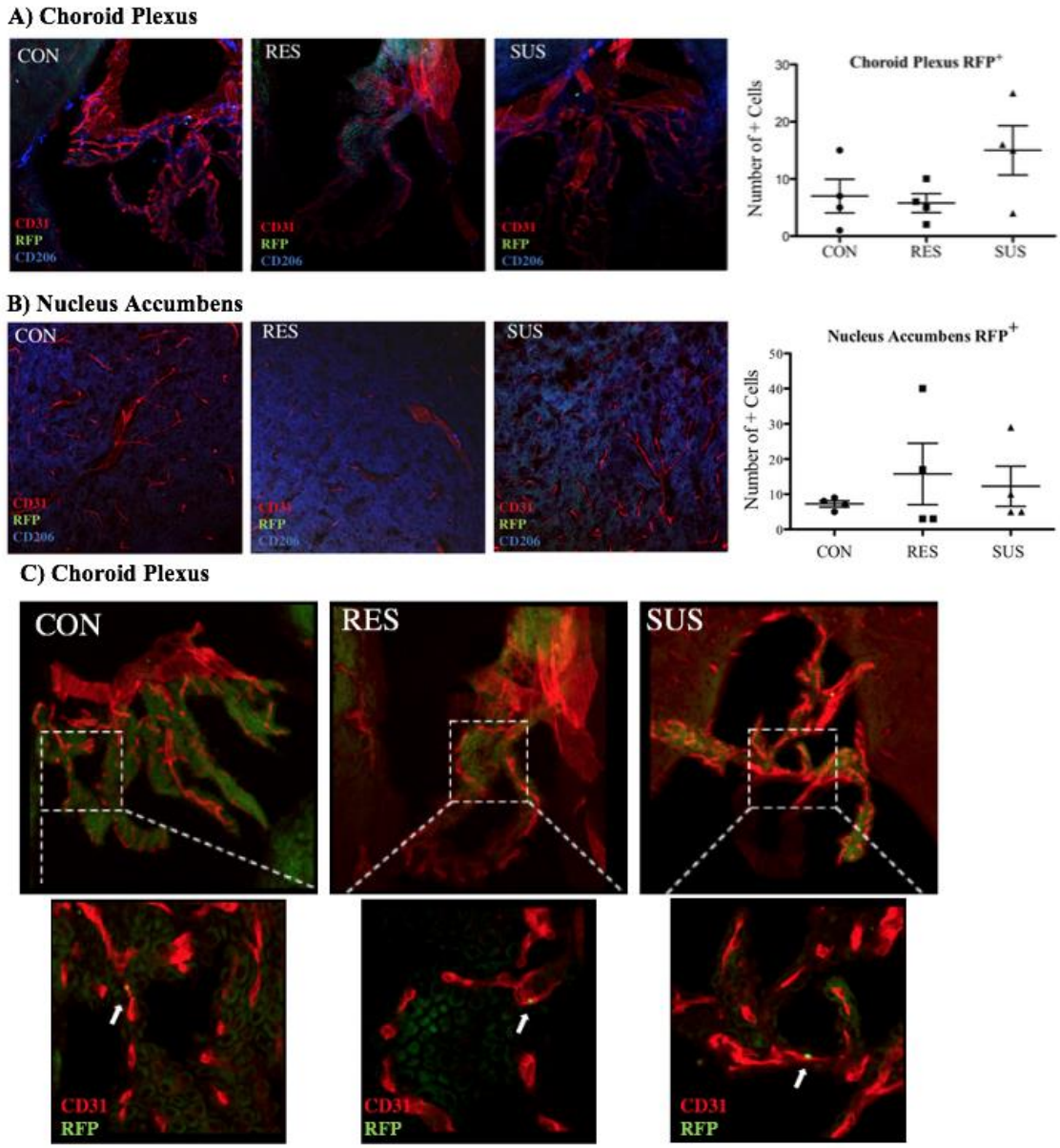


**Figure 3. Characterization of peripheral immune cell infiltration into the brain after chronic social defeat stress**. Representative image of each group (n=4). Sections were stained for CD31 positive cells labeled blood vessel (red), CD206 resident microglia (blue), ccr2 RFP peripheral monocytes (green), and captured at 20× magnification. Graphs show the average of ccr2$^{RFP}$ positive cells ± SEM. (A) Choroid plexus sections from transgenic *ccr2*$^{RFP}$:: *cx3cr1*$^{GFP}$mice. (B) Nucleus accumbens sections from transgenic *ccr2*$^{RFP}$:: *cx3cr1*$^{GFP}$mice. (C) Choroid plexus sections from transgenic *ccr2*$^{RFP}$:: *cx3cr1*$^{GFP}$mice. Sections were stained for CD31 positive cells labeled blood vessel (red), ccr2 RFP peripheral monocytes (green) and captured with 20× and 40× oil immersion lenses.

## 4. Discussion

Our results confirmed that CSDS is an effective model of stress-inducing depressive-like behavior in mice. At the end of the CSDS protocol, half of the experimental animals displayed social avoidance in the SI test, which is considered a depressive-like behavior (Golden et al., 2011). This proportion similar is similar to that of previous experiments using the same strain of mice (Ménard et al., 2017; Wang et al., 2018). Additionally, we found a general reduction of cldn5 mRNA expression in the nucleus accumbens of chronically stressed mice when compared to the levels found in non-stressed mice. The nucleus accumbens is a key structure in the mesolimbic dopamine circuit and its malfunction has been related to the anhedonia present in depressive patients (Heshmati & Russo, 2015). As social avoidance can be reflecting a loss of interest in social reward, we expected to find greater loosening of the BBB in SUS mice when compared to RES mice. However, we did not find differences between these two groups and both RES and SUS mice had decreased expression levels of cldn5 mRNA.

Knowing that that reductions in cldn5 levels have been linked to a loosening of the BBB and increased permeability (Günzel & Yu, 2013), we aimed to determine whether this increased permeability was promoting an increase in traffic of peripheral immune cells into the CNS parenchyma. We performed an immunohistochemical characterization of the infiltration of peripheral monocytes into the nucleus accumbens and the choroid plexus of non-stressed and stressed mice. We also decided to study the choroid plexus because it is considered one of the most permissive brain structures to the entrance of peripheral immune cells into the brain (Schwartz & Baruch, 2014) and it has been shown to increase its permeability even further under elevated pro-inflammatory states (Schwerk et al., 2010) as the ones induced by CSDS.

Conversely to our predictions, we did not find any differences in the number of infiltrated peripheral monocytes ($ccr2^{RFP}$ positive cells) between stressed and non-stressed animals in any of the two structures studied. We think that, given the pilot nature of the present study, which severely limits the sample size (n=4), we are missing a real statistical effect. We hope that this experiment will be repeated with greater sample sizes that yield significant results, since our initial tentative results reflect a tendency of social defeat stress to increase the number of infiltrated cells.

Lastly, we carried out an immunohistochemical characterization of the localization of the infiltrated ccr2$^{RFP}$ positive cells. A deep analysis of choroid plexus images captured with the higher magnification lens revealed that infiltrated ccr2$^{RFP}$ monocytes seem to accumulate in the perivascular space of the choroid plexus but not directly into the brain parenchyma. It appears that monocytes, rather than entering and dispersing into the brain tissue, accumulate into the perivascular space and from there influx microglia activity by the release of pro-inflammatory mediators. This finding is crucial, as it gives information about how peripheral immune cells can interact and influence the central immune system. In fact, to date, there is no agreement regarding the mechanism by which peripheral immune cells influence resident microglia (Herz et al., 2017; Pinheiro et al., 2016; Wilson et al., 2010).

Increasing the understanding on the mediation of immunological mechanisms in social stress-induced vulnerability to mental disorders may help to find and design new therapeutic strategies. In this pilot study, we highlighted how social stress compromises BBB integrity and we characterized how peripheral infiltrated monocytes act while infiltrated within the CNS.

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# 4. GENERAL CONCLUSIONS

## 4. General conclusions

Stress has been related, in both human and animal studies, to a greater risk of displaying a SUD and drug-related behaviors (Koob & Schulkin, 2018; Sinha et al., 2011). Specifically, stress experiences under certain conditions have the potential to increase individual response to the rewarding effects of drugs of abuse (Burke & Miczek, 2015; Han et al., 2017; Montagud-Romero et al., 2018; Neisewander et al., 2012; Reguilón et al., 2017; Shimamoto, 2018). In mice, CPP, an animal model that evaluates the conditioned rewarding effects of drugs of abuse, has been used to study this phenomenon. Studies repeatedly show that stress experiences increase the conditioned rewarding effects of cocaine, namely by enhancing the potential of the drug to establish drug-context memories (Montagud-Romero et al., 2018). This effect is extremely important during drug-cessation, as the exposure to contextual cues is considered one of the three most important causes of relapse, together with stress and contact with the drug (Stewart et al., 2008).

Thus, the principal objective of the present Thesis is to determine if the immune system, specifically an inflammatory response, is mediating this relation between stress experiences and an enhanced cocaine response. To validate this inflammation theory of stress-induced vulnerability to drug addiction, we set out to study social stressors, which are considered the most relevant source of stress in human being.

### Validation of social defeat's potential to induce a phenotype of long-term vulnerability

In the present Thesis, we decided to use the repeated social defeat (SD) protocol for the modeling of social stress in mice. As we previously introduced, this paradigm is widely used to mimic human social confrontations in territorial animal, such as rodents (Björkqvist, 2001; Selten et al., 2013). In this regard, an early objective of the present Thesis was to assess if this protocol was effective in promoting a stress response that could alter an individual's response to cocaine. Our results confirmed that animals that experience SD display a long-term increase in anxiety behavior measured in an open field (**Study 1**), being this phenotype replicated in other studies using the elevated plus maze (**Studies 2-5**). We also found that these defeat experiences also promoted

locomotor cross-sensitization, or in other words, an amplified locomotor response to cocaine effects (**Studies 1 and 3**).

Lastly, we confirmed that four episodes of SD stress induced a long-term enhancement of cocaine rewarding properties evaluated in the CPP (**Studies 1-6**) and in the self-administration paradigm (**Study 3**). Defeat experiences increased the sensitivity to the conditioned rewarding effects of cocaine, as stressed animals developed CPP with a dose (1mg/kg) that is not effective in animals under homeostatic conditions (Montagud-Romero et al., 2018). In the self-administration test, stressed animals consumed more cocaine than non-stressed mice and were also more prone to reinstate self-administration behavior in response to a priming dose of the drug (**Study 3**).

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Conclusion. Repeated SD protocol induces a long-term effect over mice behavior. It promotes a phenotype of vulnerability characterized by anxiety-like behavior and sensitization to the motor and rewarding effects of cocaine.

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**Environmental interventions for the management of SD consequences.**

Having demonstrated that SD experiences amplificated a subject's response to cocaine, we aimed to determine if specific environmental interventions could reverse these effects. We evaluated three environmental conditions that traditionally have been demonstrated to be useful in managing the deleterious effects of stress: social enrichment, environmental enrichment (EE) and physical exercise.

The fist intervention, social enrichment, was based on the strategy of creating environmental conditions that enhance positive social contacts between conspecifics by housing male animals in pairs, or by pairing a male with a female. These social environments proved to be an excellent intervention, which not only blocked stress effects over cocaine response, but also prevented the development of anxiety-like behavior (**Study 4**). In agreement with these results, human social support, a phenomenon that we were trying to mimic, has been repeatedly demonstrated to improve therapeutic outcomes in drug addiction treatment (Litt et al., 2016).

Conversely, environmental intervention based on increase physical exercise failed to prevent stress-induced enhancement of cocaine reward. We found that voluntary wheel

running (VWR) was rewarding for mice, which developed CPP for the compartment associated with an active running wheel. Moreover, if presented concurrently to an effective dose of cocaine, this rewarding effect was strong enough to compete against the drug to establish context memories. However, the introduction of running wheels as an environmental intervention, not only failed to prevent the sensitization of stressed mice to cocaine effects, but also enhanced the response of non-stressed mice that developed CPP induced by a subthreshold dose (**Study 6**).

Similar results were obtained with the intervention based on EE, where again both stressed and non-stressed animals developed CPP. In this environmental intervention, animals were housed with items such as tunnels or houses. However, this enhanced sensory and cognitive stimulation also failed to buffer against the anxiogenic effects of stress (**Study 5**).

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Conclusion. Environmental interventions differently modify stress behavioral consequences. Among the three interventions studied, social enrichment is the only effective strategy to prevent the development of anxiety-like behavior and cocaine CPP in stressed mice. In turn, physical exercise and EE not only fail to counteract SD effects, but also enhance the response to cocaine of non-stressed mice.

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**Neurobiological mechanisms that contribute to the enhancement of the rewarding properties of cocaine induced by social stress: neuroendocrine response**

A great number of studies in the literature have linked stress consequences with the activation of the neuroendocrine HPA axis. For instance, the appearance of anxiety-like behavior has been linked to the activation of the extended amygdala by the hormonal products of the HPA axis (Zorrilla et al., 2014). In turn, the increase of drug effects is more related with augmented DA activity in the reward system, being this effect induced by CRF released from the HPA axis (Boyson et al., 2014; Haass-Koffler & Bartlett, 2012; Han et al., 2017; Sinha et al., 2011).

Our results confirmed that social stress promoted a strong neuroendocrine response of the HPA axis. Corticosterone, which is the final hormonal product of the HPA axis, was dramatically increased in SD animals when compared to the levels of non-defeated animals (**Studies 5 and 6**).

Considering this, we wondered if the blockade of this hormonal stress response could prevent SD stress behavioral consequences. To assess that, we used a pharmacological approach by administering CRF receptor antagonists. We found that the blockade of the CRF1 receptor impeded the HPA neuroendocrine stress cascade, and as a consequence, prevented the enhancement of anxiety and cocaine response. However, the blockade of CRF2 receptors promoted the opposite effects, amplifying stress consequences (**Study 1**). In fact, it seems that while CRF1 receptors promote the initiation of the endocrine stress response, the activation CRF2 receptors starts the phase of recovery of the stress response (Reul & Holsboer, 2002).

Nevertheless, we suspected that, in addition to this direct modulation of the HPA axis over the reward system, other mechanisms must have been necessarily participating. In fact, environmental interventions based on physical activity and EE decreased corticosterone response to stress (**Studies 5 and 6),** although this effect did not revert the enhancement of cocaine's rewarding effects.

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Conclusion. The increase in the conditioned rewarding potential of cocaine is dependent on the correct activation of the neuroendocrine stress response of the HPA axis. The blockade of this cascade at pituitary level through the administration of a CRF1 receptor antagonist prevents the long-term negative consequences of stress. Conversely, the blockade of CRF2 receptors amplifies the endocrine stress response, acting as a pharmacological stressor.

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**Neurobiological mechanisms that contribute to the enhancement of the rewarding properties of cocaine induced by stress: immune system and inflammation**

We speculate that, in addition to a direct action of stress hormones over the mesolimbic reward pathway, an enhanced response to cocaine could also be explained by stress actions over the immune system. In fact, the confirmation of the necessary participation of the HPA axis endocrine response was a great boost to our hypothesis, as the HPA axis directly modulates the immune function and vice versa.

In the present Thesis, we were able to confirm that social stress induced an activation of the immune system characterized by an acute inflammatory response (**Studies 2, 4, 5, 6**). We detected enhanced levels of the pro-inflammatory cytokine IL-6 after the first and fourth SD episode in the plasma, but also in central structures such as the striatum,

prefrontal cortex and hippocampus. These levels returned to normality after three weeks, only remaining elevated in the hippocampus (**Study 2**). Additionally, we also found that SD stress induced a long-term sensitization of the immune system, turning it into a primed state, increasing its reactivity to further immunological challenges. And in this hypersensitive state, SD animals displayed an exacerbated inflammatory response to cocaine administration during the CPP protocol (**Study 2, 4, 5, 6**).

Then, through the administration of an anti-inflammatory drug (indomethacin) prior to SD episodes, we found that the attenuation of the inflammatory response prevented the sensitization of both the reward and the immune systems. However, conversely to our predictions, we failed to find a mediation of inflammatory processes in the genesis of stress-induced anxiety (**Study 2**). Probably, this discrepancy is due to the fact that the enhancement in cocaine response and in anxiety behaviors is sustained by different mechanisms, being the brain structures that sustain anxiety behavior more sensible to inflammatory challenges (Wohleb et al., 2011, 2013, 2014).

In parallel, in **Study 7** we carried out, in collaboration with Dr. Russo laboratory, a pilot study that aimed to increase the knowledge on the cellular and molecular mechanisms that may be mediating the impact of SD stress over the immune function and over the vulnerability to display stress-related disorders. We found that, together with the inflammatory response, chronic SD also disrupted the CNS vascular system by decreasing the expression of cldn5 and by promoting the loosening of the BBB (Günzel & Yu, 2013). Additionally, we performed an immunohistochemical characterization of the infiltration of peripheral immune cells into the CNS, finding that infiltrated cells, rather than entering directly into the brain tissue, accumulate into the perivascular space. This finding was crucial since it clarifies the mechanism through which peripheral immune cells can interact and influence central immune system and communicate inflammatory signals.

Conclusion. We validate the inflammation theory of stress-induced vulnerability in drug addiction. SD stress induces an acute inflammatory response and posterior sensitization of the immune system. This response is mediating the long-term increase in cocaine reward induced by SD, as treatment with an anti-inflammatory drug prevents its appearance. SD stress can also impair BBB's permeability, exposing the brain to peripheral immune components that are captured in the perivascular space.

**Integration of the neuroendocrine system and the immune system in the enhanced sensitivity to cocaine. The vicious circle model**

The potential of social stress to stimulate the immune system to release inflammatory cytokines is key to understand the progression of cocaine addiction and its chronicity. As we explained in the introductory section this Thesis, pro-inflammatory cytokines such as IL-6 directly stimulate the HPA axis (Silverman et al., 2004). In succession, this stimulation eventually increases stress hormones, which in turn increases DA function through acting in the VTA (Boyson et al., 2014). Thus, long-term enhancement of inflammatory markers induced by stress would alter the function of the reward pathway, causing long-term neuroadaptations (Haass-Koffler & Bartlett, 2012) that would lead to an increased hedonic response to cocaine (Han et al., 2017). These relations conform a vicious circle between neuroendocrine stress response, sensitization of the reward pathway and inflammation (see Figure 5). The sensitization of the reward pathway may increase the probability to repeat contact with cocaine, which as we have demonstrated (**Study 2**) would amplify the existing inflammatory states even further. This, together with the fact that during drug abstinence there is a strong neuroendocrine stress response (Koob & Volkow, 2010; Tunstall & Carmack, 2016), would close the circle.

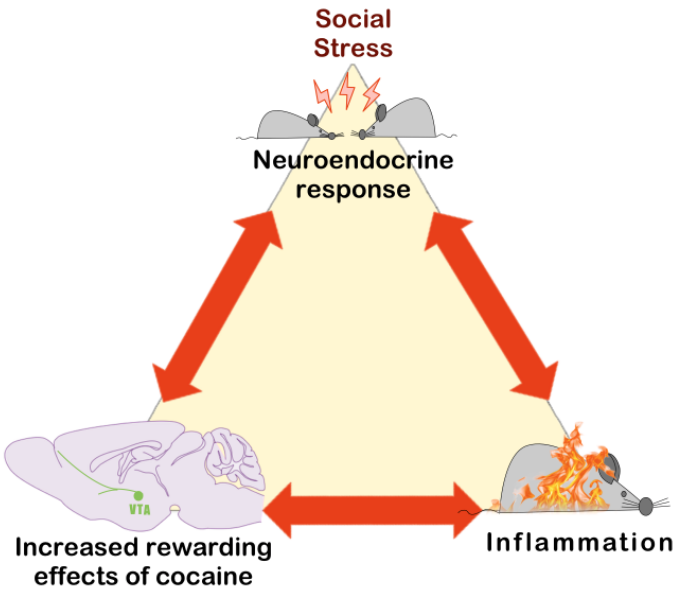


**Figure 5. Vicious circle model.** The figure depicts a possible explanatory model of stress enhanced vulnerability to cocaine effects based on a reciprocal cause and effect between neuroendocrine stress response, inflammation and increased sensitivity to cocaine reward.

**The vicious circle model explains the effectiveness of environmental and pharmacological treatments.**

***Social enrichment***

In the present Thesis, we found that there is an increase of Oxytocin (OT) in animals housed under social enrichment conditions (**Study 4**). This neuropeptide, which is released during physical contact (Carter, 2003), has been reported to exert anti-inflammatory effects (Li et al., 2017; McQuaid et al., 2014). We stated that the buffering effects of social interventions are linked to its potential to increase OT levels and, eventually, to decrease inflammatory states triggered by SD. In fact, we found that socially enriched animals did not display an exaggerated inflammatory response to cocaine administration in the CPP, which means that their immune system was not primed. Additionally, social interaction is rewarding and has the potential to act as an alternative reward to drugs (Zernig et al., 2013). Therefore, being housed in a positive social environment can break the abovementioned "vicious circle" (see Figure 6), mainly through its anti-inflammatory properties.

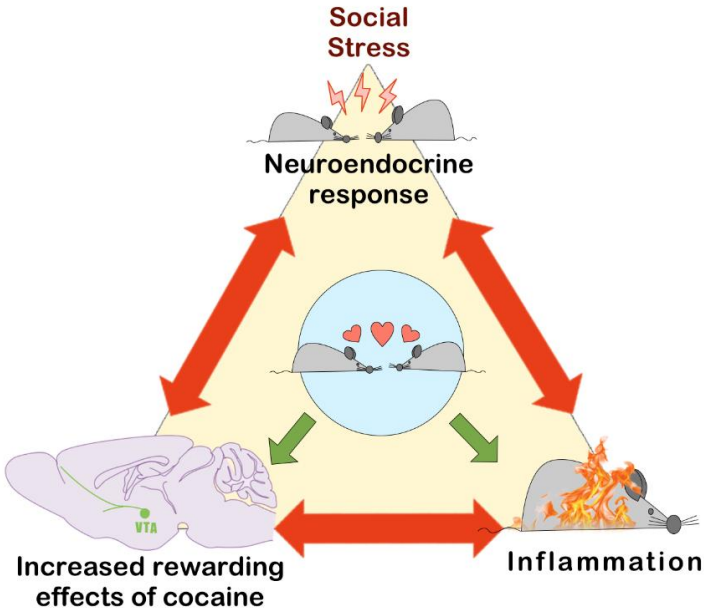


**Figure 6. Social environmental interventions break the vicious circle.** The figure depicts a possible explanatory model of the effectivity of social interventions. Social enrichment protects against the enhanced vulnerability to cocaine through its anti-inflammatory effects and by preventing the sensitization of the reward system.

It should be stressed that OT's protective actions are not as simple as a linear effect. We have studied two different social set-ups: housing in pairs with a female or with a familiar conspecific since adolescence. While both social interventions were effective, only in the condition of being housed with a female were plasmatic OT levels significantly elevated. However, for the appearance of social buffering effects, OT activity is necessary, because the chronical antagonism of its receptors with Atosiban reversed the protective effects of being housed with a familiar conspecific (**Study 4**).

Moreover, pre-treatment with exogenous OT also proved to be effective in preventing the long-term effects of stress over anxiety and cocaine response. Administered prior to SD episodes, it prevented the appearance of cross-sensitization to cocaine locomotor effects and CPP induced by a non-effective dose of the drug. When using effective drug doses, OT pre-treatment also decreased the number of required sessions to extinguish drug memories in the CPP and in the self-administration paradigm (**Study 3**). This acceleration of the extinction of drug memories could be also sustained by the fact that pre-treatment with the neuropeptide OT enhanced BDNF expression within the striatum and the hippocampus, structures deeply related with associative learning processes (Novkovic et al., 2015; Ohline et al., 2019).

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Conclusion. Social environmental interventions can break the vicious circle. This social buffering is dependent on the neuropeptide OT, which exerted anti-inflammatory actions. The administration of exogenous OT exerts a similar protective effect, preventing the sensitization of the reward system and accelerating the extinction of drug-related memories.

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***Environmental Enrichment and physical exercise***

As previously commented, environmental interventions based on EE or physical exercise failed to show a protective effect against the effects of SD over cocaine response. Even though both were effective in decreasing corticosterone response to SD stress, this effect was not enough to break the vicious circle (see Figure 7). Moreover, both interventions enhanced the increase of inflammatory parameters induced by SD (**Studies 5 and 6).** This inflammatory effect was particularly dramatic in animals under physical exercise intervention since both stressed and non-stressed mice presented elevated striatal and hippocampal levels of the inflammatory cytokine IL-6.

Lastly, both interventions promoted an amplificatory effect of cocaine reward as control animals also developed CPP by a dose that was not effective in non-stressed animals housed in standard conditions. We think that this effect is supported by the fact that these interventions promoted an up-regulation of BDNF. In fact, we confirmed these enhancements in animals in the intervention of physical exercise (**study 6).** In turn, the increase of this trophic factor has been linked to an increased cognitive performance and enhanced spatial learning (Novkovic et al., 2015; Ohline et al., 2019), which could explain why animals establish drug-associated memories more easily.

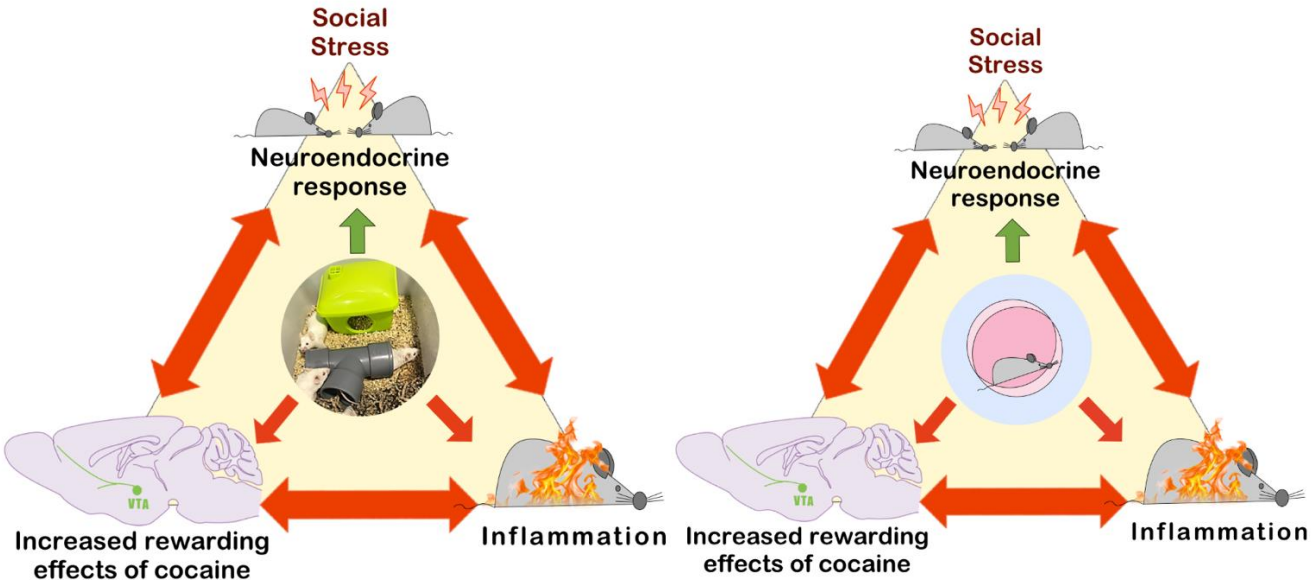


**Figure 7. Environmental enrichment and physical exercise interventions failed to break the vicious circle.** The figure depicts a possible explanatory model of the ineffectivity of these interventions. Both interventions effectively decreased the neuroendocrine response to stress. However, they amplificated the inflammatory effect of SD. They also enhanced cocaine's rewarding effects, presumably by enhancing the animals' capacity to establish drug-associated memories.

Conclusion. Interventions based on EE and physical exercise fail to break the vicious circle. Moreover, they also promote the development of cocaine CPP in non-stressed animals, presumably by enhancing the animals' capacity to establish drug-associated memories

**Overall conclusions and value of the Thesis**

This Thesis increases the knowledge on the neurobiological bases of stress-increased vulnerability to cocaine addiction. It proposes an integrative model, "the vicious circle", which links stress modulation of different brain systems to the individual response to cocaine reward. We hope that this basic research will help, by building this explanatory model, in the development of more accurate preventive interventions, as well as better treatment protocols for the management of the influence of stress on addictive disorders.

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