Effects of maternal separation with early weaning on cocaine addictive behaviour and consequences on neuroplasticity.

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DOCTORAL THESIS UPF/2020

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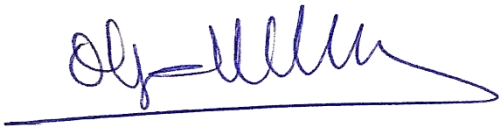

Fdo. Dra. Olga Valverde Granados

This doctoral thesis has been supported by grants from the European Union's Horizon 2020 research and innovation program 2014-2020 (Grant Agreement Nº 634143), the Spanish Ministry of Economy, Innovation and Competitiveness (SAF2016-75347-R-FEDER) and the Spanish Ministry of Health (RD/16/0017/0010-FEDER), Plan Nacional Sobre Drogas (#2014/020 and #2018/007). The Department of Experimental and Health Sciences (UPF) is a 'Unidad de Excelencia María de Maeztu' funded by the Spanish Ministry of Economy, Innovation and Competitiveness. ACZ was funded by CONACYT grant (276577) from the Mexican government.

A mis padres y mi hermana, los tres pilares de mi vida.

"Una de las trampas de la infancia es que no hace falta comprender algo para sentirlo. Para cuando la razón es capaz de entender lo sucedido, las heridas en el corazón ya son demasiado profundas."

Carlos Ruiz Zafón

"El lugar que amamos, ese es nuestro hogar; un lugar que nuestros pies pueden abandonar, pero no nuestros corazones"

"El lloc que estimem, aquesta és la nostra llar; un lloc que els nostres peus poden abandonar, però no els nostres cors"

Oliver Wendell Holmes

AGRADECIMIENTOS

Pues bueno, después de 5 años, ya estamos en la tan esperada sección de agradecimientos. Me cuesta creer que al fin esté escribiendo esto. Estos años han estado llenos de muchas experiencias, personas, lugares y anécdotas. Y qué decir del gran crecimiento profesional, pero sobre todo el crecimiento personal que he experimentado.

Desde muy pequeña tenía claro que quería ser científica. Me imaginaba en un laboratorio lleno de matraces, investigando e inventando cosas nuevas. A decir verdad, este camino ha sido muy divertido, pues la mayoría de la gente que quiero y está a mi alrededor, sabe que amo y me apasiona lo que hago. Pero claro, la ciencia no siempre es agradecida (en realidad la mayoría del tiempo) y nos otorga fracaso tras fracaso hasta que al fin, un día, revelamos un western blot y ¡ahí está!, ¡la señal tan buscada!

Ahora bien, este camino de subidas y bajadas científicas no hubiese sido el mismo si no hubiera tenido a mi maravillosa familia científica GReNeC. En varias ocasiones les he dicho lo genial que es despertar entre semana y sentirte con todo el ánimo de ir a trabajar, pues sé que en el despacho 317 (…y 325, obviamente), me espera un día lleno de risas aseguradas con personas completamente admirables. Y a estas personas no las hubiera encontrado si no hubiera sido por Olga.

Olga, aún recuerdo cuando vine a verte por primera vez a tu despacho para plantearte la idea de hacer el doctorado en tu laboratorio. Fue tan agradable encontrarme con una sonrisa tan franca y amable desde el minuto cero. Recuerdo que me escuchaste y me dijiste que me apoyarías

para poder venir a Barcelona, y así fue. Quiero agradecerte toda tu guía y confianza en mí, y por hacerme sentir una más desde el primer día que vine; por dejarme ir re-explorando y replanteando el proyecto, pero sobre todo te doy las gracias por la calidad de persona que eres, pues ves a tu equipo de trabajo como eso, un equipo.

Y bueno, que puedo decir de este gran equipo GReNeC, que siempre está al pie del cañón para apoyarse uno a otro y sacar adelante los proyectos, pero no se limita a eso, también sabemos organizar beer sessions épicas.

Y como ya he dicho, no solo he encontrado en ese laboratorio, grandes colegas, también he hallado a amigos incondicionales. **Ana,** ¿qué te puedo decir a ti que no lo sepas ya? Gracias infinitas por tu eterna paciencia conmigo, por tener las palabras precisas cuando se necesitan; por las tardes de viernes de estadística inolvidables. Por esas veces que hemos chocado las manos alegrándonos de un resultado (que ya habíamos celebrado miles de veces jaja). Por toda la complicidad que tenemos, tan es así que solo tú entenderás esto "sex and rearing". Gracias por ser una amiga incomparable, por las charlas eternas, las tardes de chicas y sephora, y por los gin tonics imaginarios (y los que no) que hemos tenido contándonos el cotilleo del día.

De la mano de Ana, viene **Alba,** la tercera integrante del equipo AAA. Mi chiquis, eres una persona que desde el primer momento me sorprendió por tu gran inteligencia, temple y madurez. Gracias por todas esas veces que me ayudaste a encontrar soluciones a varios resultados que me costaban la vida entender, pues siempre me haces ver una cara de la moneda que pasaba desapercibida para mí. Y bueno,

también gracias por llevarme nuevamente al lado oscuro musical jaja. Gracias por tantos buenos ratos que hemos tenido en Barcelona y Badalona, y por ser la amiga tan increíble que eres.

Neus, que buenos recuerdos tengo contigo y el laboratorio. Ese momento de freezing total al lado de la balanza por culpa de un anticuerpo jaja. Y lo mejor de todo fue que la relación entre nosotras no quedó solo ahí. Ha sido muy bello tenerte por amiga, dejarme conocerte y entender un poquito más de ti. Ten la seguridad de que siempre tendrás en mí a una amiga.

Pasando al equipo de las rubias, tenemos a Sandra y Lidia. **Sandra,** mi rubia sexy, eres una persona totalmente especial en mi vida y la mejor compañera de gimnasio que he tenido. Cuantos momentos hemos pasado siendo yo la "potrosa" que a partir de las 16:00 comenzaba a rondar tu sitio. Gracias infinitas por tu gran capacidad de empatizar conmigo y escucharme; por los consejos, los regaños y todos los ánimos que siempre me has dado. Gracias por siempre tener una sonrisa incondicional, incluso cuando "*el peeeeu*" era de un tamaño XL. Que orgullosa debe sentirse la terreta sollanense (que también es mía) al tenerte ahí como hija pródiga.

Lídia, de ti admiro un montón de cosas…tu templanza, serenidad, tu capacidad para hacer de todo y en cada una de las cosas entregarte al 100%. Muchas gracias por ser una compañera y amiga tan única, por esa benevolencia que siempre expresas en cada uno de tus actos y por entrar en modo locura cuando se requiere (entiéndase momentos cutre bar, karaokes y posteriores jaja). A ti, **Laia,** gracias por enseñarme la capacidad de ser multifacética…así como por la mañana haces una CPP,

por la tarde bailas, educas niños, diriges la muixeranga, tocas guitarra, escribes en 1 semana un súper libro para Magdalena. Me encanta tu capacidad de adaptación a las situaciones, aunque siempre mantienes tu sello personal.

Miguel Ángel, a ti primero perdón por haberte robado tu sitio (que siempre me dijiste que pensabas que cuando se fuera Irene este lugar sería para ti jaja). Mil gracias por la cantidad de datos curiosos que nos enseñaste, desde historia, física, vídeos raros en youtube, memes random, hasta los papers más recientes y novedosos sobre los temas del laboratorio. Gracias por compartir mi gusto por el picante y las micheladas, porque así me pudiste recomendar varios restaurantes muy guais en Barcelona.

Y para nuestra más reciente adquisición en el laboratorio, también tengo cosas por decir. **Inés,** gracias por demostrarme que la sonrisa es una de las llaves que más puertas abre. De verdad que te agradezco todos los grandes momentos que hemos compartido en el laboratorio y fuera de él. Por compartir una curiosidad científica a la par de la mía, desde saber qué pasa cuando el hielo seco se sublima, preparar "agua de mar", medir el pH con protección jaja, imaginar como una mosca va a 200 km/h dentro de un coche, el ponernos a buscar el significado de los nombres de los meses del mes, o bien, el cómo chocar la mano correctamente mirando el codo, entre miles de datos más. Chiquitina, gracias por ser tan asertiva y por haberme ofrecido tu amistad en tan poco tiempo, así sin más.

A ti **Xavi**, gracias por mantener en orden y con toda la "seguridad y prevención" el laboratorio. Por entender mi TOC en poner los potes de

menor a mayor y las pipetas a su máximo volumen jaja. También, gracias por tantas risas que nos das con los temas que son tan tú. Eres un técnico y un amigo muy chingón…visca Catalunya!

Y aunque estos años he sido muy feliz en Barcelona y he encontrado a los mejores amigos que me hubiese podido imaginar, siempre tengo una parte de mí que está donde mi familia. No importa que exista una distancia física de más de 9000 kms, nuestros corazones siempre han permanecido conectados. Sin ustedes, familia, no estaría en donde ahora estoy.

Mamá, gracias porque desde muy pequeña supiste identificar mi deseo de ser científica y siempre me motivaste para alcanzarlo. Recuerdo que me contaste que un día siendo yo muy peque, encontré en casa de mi abuela un libro de química y que en cuanto lo abrí, pudiste ver que mis ojos se llenaban de asombro al ver los dibujos, los matraces, a las personas vestidas con batas y que me brotaron un montón de preguntas. Y aunque no siempre tuviste las respuestas para todas ellas, siempre me ayudaste a buscar las respuestas. Gracias por ser la persona que más ha creído y confiado en mí; por apoyarme a dar pasos que yo sola no me hubiera atrevido (como esa vez de pedir la beca en el Tec, que para mí era solo una ilusión que te contaba mientras comíamos tortas jaja). Gracias porque a pesar de la distancia, has estado para mí en momentos muy duros y de muchas dudas. Gracias por que cada que nos vemos, parece que el tiempo no pasa y podemos volver a reírnos, jugar videojuegos, irnos a dar la vuelta y por un elote jaja. Gracias mami, por todo el amor que siempre nos has demostrado a mí y a mi hermana y porque cuando estoy en México, haces de todo para que me sienta como si nunca me hubiese ido.

Papá, eres uno de mis soportes vitales en esta vida y en este proceso de crecimiento personal y profesional. Gracias por enseñarme el significado de resiliencia, de seguir adelante a pesar de las adversidades. Creo que estos años fuera, me han permitido acercarme a ti desde otra perspectiva; contándote cosas que jamás me hubiese atrevido y lo más maravilloso, es que he encontrado en ti las palabras más bellas, con más amor y sabias que he podido imaginar. Gracias por mirarme con esos ojos de admiración y orgullo, espero de verdad algún día poder estar a la altura de todo eso que piensas y crees de mi y poder devolverte un poco de lo mucho que tu me has dado. Gracias por siempre estar involucrado en mi trabajo y en ver como va la investigación y los "ratones" jaja.

Sandy, hermanita…cuantas cosas tengo que decirte a ti, pero ahora las resumiré. Quiero que sepas que desde el primer momento en que te vi, supe que ahora tenía una razón para ser la persona más fuerte de este mundo y para quien debía ser un ejemplo. Y si, sin lugar a dudas, hasta la fecha, el solo hablar contigo por breves minutos o intercambiar mensajes escritos, me dejas con una fuerza descomunal. Gracias por motivarme a ser mejor ser humano, a ser mejor hermana, mejor hija, mejor profesionista. Te admiro con locura y sé que tienes todo para llegar hasta donde tú te propongas.

Fernando, gracias por acompañarme a lo largo de este gran viaje profesional, que creo que comenzó desde que te conté que haría el verano de la ciencia con fructanos jaja. Gracias infinitas por toda tu paciencia, por escucharme cuando he necesitado hablar con alguien, por tus consejos, por tu apoyo y por todo el cariño que le has demostrado desde siempre a mi familia. Gracias por que siento que aunque yo no

esté en México, tengo a una extensión de mi allá para no tener que ser siempre la hija ausente, puesto que tu me has ayudado a dar muestras de amor a mis padres y a mi hermana de que sigo ahí. Gracias por la amistad más pura, sincera e incondicional que siempre me has brindado.

Y desde luego que gracias a todos los que han estado en este camino…a mis primos **Omar** y **María José**, porque a pesar de que yo soy mayor que ellos, muchas veces me han permitido ser la prima menor. A mi prima **Fany** y a mi tía **Paty** porque aún en la distancia, me han acompañado echándome todos los ánimos del mundo. Y también gracias a los que ya no están, pero que al inicio de este proyecto me mostraron el camino a seguir…a mi tío **Baltazar** que tantas veces me habló tan hermosamente de Barcelona y que me mostró una foto de él en el monumento a Colón y me dijo con voz de profeta "ahí estarás un día". A mi abuelita **Pifas**, que siempre me decías que "se me iba a quemar el cerebro de tanto estudiar"; siempre te llevaré conmigo y siempre seguiré esperando ese momento de volver a Ébano y verte sentada en la hamaca con una sonrisa feliz de vernos llegar a casa.

Gracias también mis amigos de Barcelona que han estado en las buenas pero sobretodo en las malas (Augusto, Marapau, Andrés, Eve, Vincent, César). Sin ustedes, esto no hubiera sido lo mismo.

¡Prometo que estoy por terminar! Gracias infinitas a todos y cada uno de ustedes por acompañarme en este camino y por demostrarme que las buenas personas están por todos lados; por enseñarme que aunque en algunas ocasiones me he sentido dividida, todo esto ha valido la pena, puesto que ahora tengo dos sitios a los cuales puedo llamar hogar.

ABSTRACT

Chronic exposure to stress, especially in early life, has been associated with the onset and the severity of several psychiatric disorders in adults. Moreover, early-life stress induces maladaptive long-lasting brain effects that increase the likelihood of developing substance use disorders or depression. It is known that early-life stress affects in different way women and men, however, this phenomenon is poorly explored. Understand the mechanistic connections among early-life stress and development of substance use disorders and depression, could help to develop new therapeutic targets against these public health problems. Here, we sought to investigate the effects of early-life stress in cocaine addiction behaviour and the molecular alterations induced by both factors, cocaine exposure and early stress. For this reason, we tested the impact of early-life stress induced by maternal separation with early weaning in CD1 male and female mice at different phases of cocaine self-administration. We also investigated in brain regions associated with stress, reward and impulsivity, the subsequent alterations on AMPA receptor subunit composition and other molecules associated with neuroplasticity process. Our results yield that maternal separation with early weaning has behavioural effects in males while females appear to be resilient to this kind of early-life stress. Additionally, maternally separated males express despair-like behaviour, higher cocaine intake, increased vulnerability to the acquisition of cocaine self-administration and incapacity to extinguish the cocaine self-administration behaviour. Molecular analyses of ventral tegmental area, nucleus accumbens and medial prefrontal cortex show sex-induced alterations in the composition of the AMPA receptor but also alterations after cocaine

exposure. Maternal separation with early weaning and cocaine exposure also alters the expression of GluA1, GluA2, pCREB and CREB in these brain areas.

Altogether, our results displayed changes in neuroplastic molecules that play a crucial role in depression and the regulation of the rewarding effects of cocaine, helping to elucidate the mechanisms involved in the progression from cocaine use to cocaine abuse in both women and men.

RESUM

L'exposició crònica a l'estrès, particularment durant els primers anys de vida, s'ha relacionat amb l'aparició i amb la gravetat de varies malalties psiquiàtriques en a l'etapa adulta. A més, l'estrès de la vida primerenca indueix efectes cerebrals que poden perdurar fins a l'edat adulta augmentant la probabilitat de desenvolupar trastorns d'ús de substàncies i depressió. L'estrès de la vida primerenca afecta de forma diferent a homes i dones, malgrat això, aquet fenomen roman poc explorat. Entendre els mecanismes que connecten l'estrès de la vida primerenca i el desenvolupament de l'addicció i depressió, pot ajudar a la creació de noves teràpies contra aquests problemes de salut pública. En aquest treball, es pretén investigar els efectes de l'estrès de la vida primerenca sobre l'addició a la cocaïna i les alteracions moleculars provocades per ambdós factors, cocaïna i estrès. Per aquest motiu, es va estudiar l'impacte de l'estrès induït per la separació maternal amb deslletament precoç en ratolins CD1 mascles i femelles a diferent fases de l'auto-administració de cocaïna. També s'han investigat les alteracions a la composició del receptor AMPA i altres molècules relacionades amb la neuroplasticitat a regions cerebrals que modulen l'estrès, la recompensa i la impulsivitat. Els nostres resultats mostren que la separació maternal amb deslletament precoç té efectes al comportament dels mascles mentre que les femelles semblen ser resistents a aquest tipus d'estrès de la vida primerenca. A més, els mascles separats de la mare mostren un comportament semblant a la depressió, consumeixen més cocaïna i expressen major vulnerabilitat a l'adquisició de l'addicció a la cocaïna, així com una incapacitat d'extingir el comportament d'auto-administració de la droga.

Els anàlisis moleculars de l'àrea tegmental ventral, el nucli accumbens i l'escorça prefrontal medial mostren alteracions induïdes pel sexe en la composició del receptor AMPA, però també alteracions degudes a l'exposició a la cocaïna. La separació maternal amb deslletament precoç i l'exposició a la cocaïna també alteren l'expressió de GluA1, GluA2, pCREB i CREB en aquestes zones cerebrals.

En conjunt, els nostres resultats demostren canvis en les molècules neuroplàstiques associats amb la depressió i la regulació dels efectes gratificants de la cocaïna, ajudant a dilucidar els mecanismes implicats en la progressió de l'ús de cocaïna a l'abús, tant en homes com en dones.

RESÚMEN

La exposición crónica al estrés, particularmente durante la primera etapa de la vida, se ha asociado con el desarrollo y la severidad de una gran variedad de trastornos psiquiátricos en la edad adulta. Además, el estrés que ocurre en la primera etapa de la vida induce alteraciones cerebrales que pueden incrementar la probabilidad de desarrollar adicción o depresión. El estrés temprano puedo afectar de diferente forma a mujeres y hombres, sin embargo, este fenómeno no se ha explorado a profundidad. Entender los mecanismos que conectan el estrés de la etapa temprana con el desarrollo de adicción y depresión, puede colaborar a la creación de nuevas terapias para tratar estos problemas de salud pública. En este trabajo estudiamos los efectos que tiene el estrés de la etapa temprana en la adicción a la cocaína, así como las alteraciones moleculares inducidas por ambos factores, la cocaína y el estrés. Por este motivo, evaluamos el efecto de la separación maternal con destete temprano en las diferentes fases de la auto administración de cocaína, en ratones CD1 tanto hembras como machos. También exploramos las modificaciones en la composición del receptor AMPA y de otras moléculas asociadas a la neuroplasticidad, en regiones cerebrales que modulan el estrés, la recompensa y la impulsividad.

Nuestros resultados muestran que la separación maternal con destete temprano tiene efectos conductuales en machos, mientras que las hembras parecen ser resilientes a esta clase de estrés. Además, los machos separados de la madre muestran una conducta similar a la depresión, mayor consumo de cocaína, mayor vulnerabilidad a adquirir la conducta de auto administración de cocaína, así como una incapacidad de extinguir esta conducta.

Los análisis moleculares del área ventral tegmental, núcleo accumbens y corteza prefrontal media muestran alteraciones debidas al sexo en la composición del receptor AMPA y también modificaciones después de la exposición a la cocaína. La separación maternal con destete temprano y la exposición a la cocaína, cambia la expresión de GluA1, GluA2, pCREB y CREB en las áreas cerebrales estudiadas.

En conjunto, nuestros resultados muestran modificaciones en moléculas asociadas a neuroplasticidad, involucradas en el desarrollo de depresión y en la regulación de los efectos reforzantes de la cocaína, ayudando a elucidad el mecanismo involucrado en el progreso de uso a abuso de cocaína tanto en mujeres como en hombres.

LIST OF ABBREVIATIONS

ACTH: adrenocorticotropin hormone

AMPA: alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AVP: arginine vasopressin

BDNF: brain derived neurotrophic factor

BSA: bovine serum albumin

Ca^{2+}: calcium

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase

CNQX: cyanquixaline

CP-AMPArs: calcium-permeable AMPA receptors

CPP: conditioned place preference

CREB: cAMP response element-binding

CRF: corticotropin-releasing factor

CRG: corticotropin-releasing hormone

CUD: cocaine use disorder

D1R: dopamine receptors 1

D2R: dopamine receptors 2

DAT: dopamine transporter

EPM: elevated plus maze

GABA: gamma-aminobutyric acid

GluA1: AMPA receptor subunit 1

GluA2: AMPA receptor subunit 2

GR: glucocorticoid receptors

Gria1: glutamate Ionotropic Receptor AMPA Type Subunit 1 gen

Gria2: glutamate Ionotropic Receptor AMPA Type Subunit 1 gen

HPA: hypothalamic-pituitary-adrenal

HRs: high locomotor responders

KOR: kappa-opioid receptor

LRs: low locomotor responders

LTD: long-term depression

LTP: long-term potentiation

MDD: major depressive disorder

Mg^{2+}: magnesium

mPFC: medial prefrontal cortex

MR: mineralocorticoid receptor

MSEW: maternal separation with early weaning

MSNs: medium spiny neurons

mTOR: mammalian target of rapamycin

NAc: nucleus accumbens

NBQX: disodium salt hydrate

NMDA: N-methyl-D-aspartate

OD: optical density

pCREB: phosphorylated form of CREB

PD: postnatal day

PFC: prefrontal cortex

PKA: protein kinase A

PKC: protein kinase C

SA: self-administration

SEM: standard error of the mean

SN: standard nest

SUD: substance use disorder

TH: tyrosine hydroxylase

TNF: tumour necrosis factor

TST: tail suspension test

VTA: ventral tegmental area

WHO: World Health Organization

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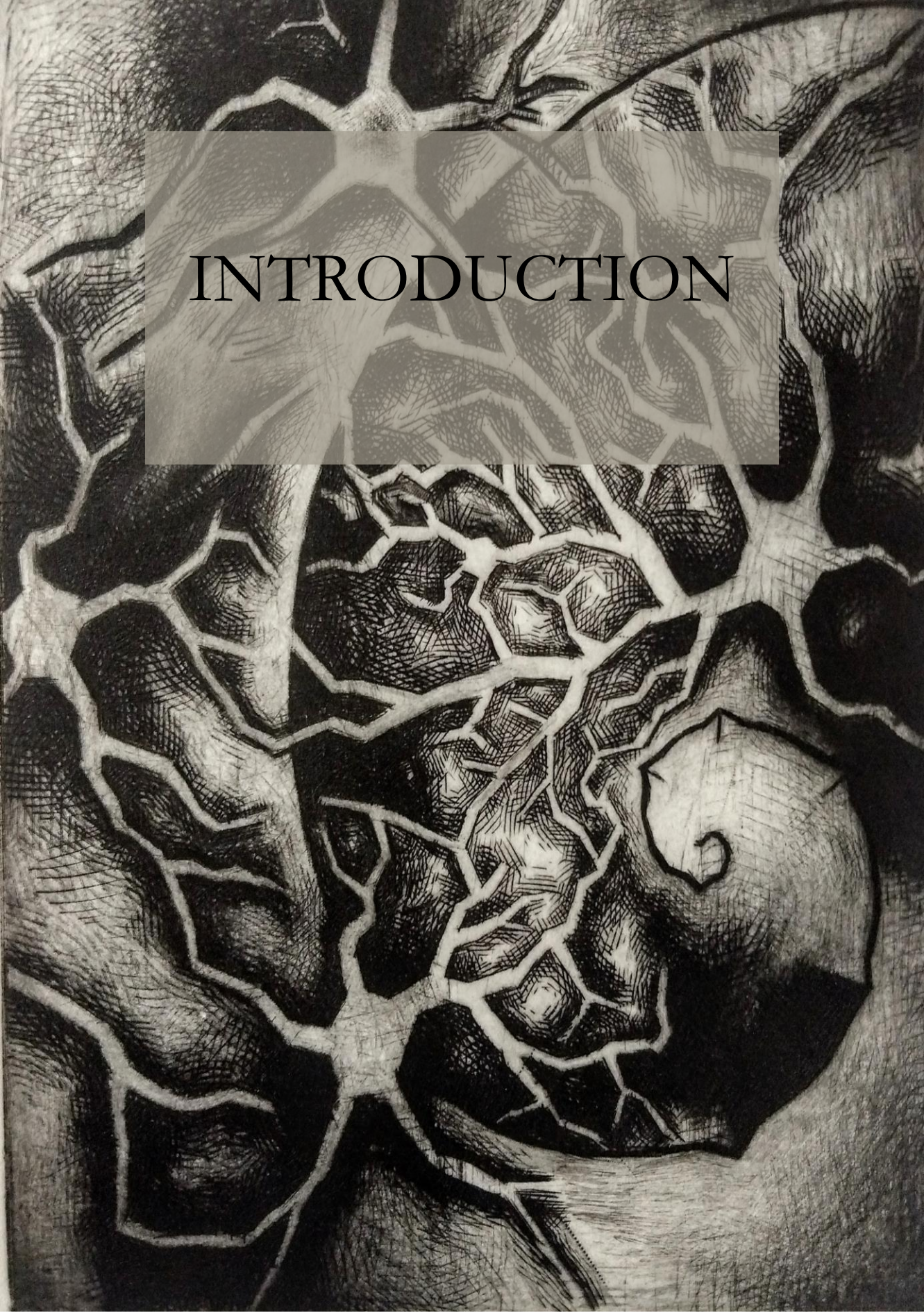


INTRODUCTION

1. Childhood: a critical period for human development

According to the Convention of the Rights of the child, a child means every human being below the age of eighteen years unless, under the law applicable to the child, the majority is attained earlier (UN General Assembly, 1989). A broader definition given by industrialising countries, childhood is a period of innocence, vulnerability and development covering since birth until adulthood, which is often set at 18 years (Evanz, 2017).

Even though the definition is just a social construction, what we really know is that childhood is a critical window of brain plasticity in the lifespan, in which the experiences will have strong effects on the behaviour and function of the neural circuits **(Figure 1)** (Hartley and Lee, 2015; Knudsen, 2004; Sylva, 1997).

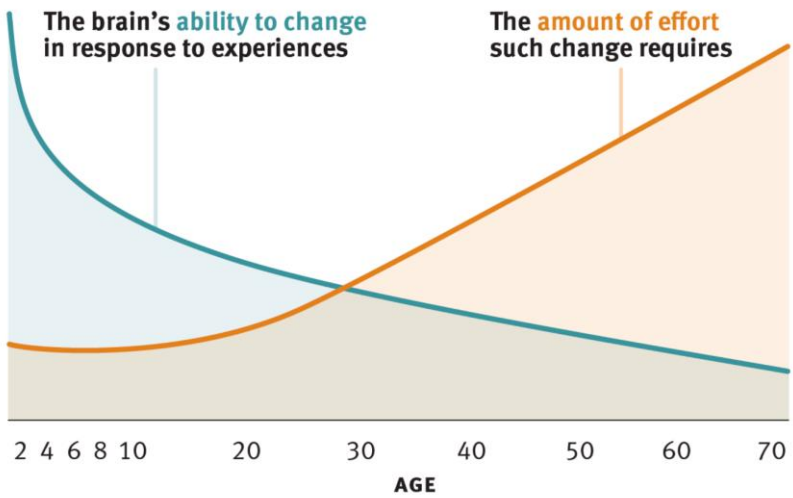


Figure 1. The ability of environmental experiences to change brain integrity and function across the lifespan. Obtained from: Brain Architecture, <www. developingchild.harvard.edu>.

Therefore, early-life experiences will have positive or negative long-lasting consequences that will affect the brain function, cognitive and emotional development (Krugers et al., 2017).

There are some conditions that must be accomplished to determine that experience will have consequences on the circuit during a critical period:

a) The data sent to the neural circuitry need to be reliable and precise to trigger the function of the circuit (Knudsen, 2004).

b) The circuitry must have a balance between excitatory and inhibitory signalling to encode the data received (at least at the onset of the critical period plasticity) (Fagiolini and Hensch, 2000; Teicher et al., 2016).

c) It has to be the activation of several mechanisms related to plasticity **(Figure 2),** for example: modification of the axonal or dendritic morphology, formation or elimination of synapses and strengthening of synaptic connections that were potentiated by the experienced, by the insertion of cell adhesion molecules in synapsis (Knudsen, 2004).

1.1. Adverse early-life experiences: child maltreatment

As mention before, adverse early-life experiences can have long-lasting effects that could persist until adulthood. Several studies have been demonstrated that adverse events during childhood, could increase the risk to develop in a future, several psychiatric disorders and also a great

variety of physical health problems (Nelson and Gabard-Durnam, 2020; Tarantola, 2018).

Adversity is defined as a violation of the expectable environment that takes the form of biological hazards, psychosocial hazards, of complex exposures of both hazard types, with negative effects on development (Nelson and Gabard-Durnam, 2020).

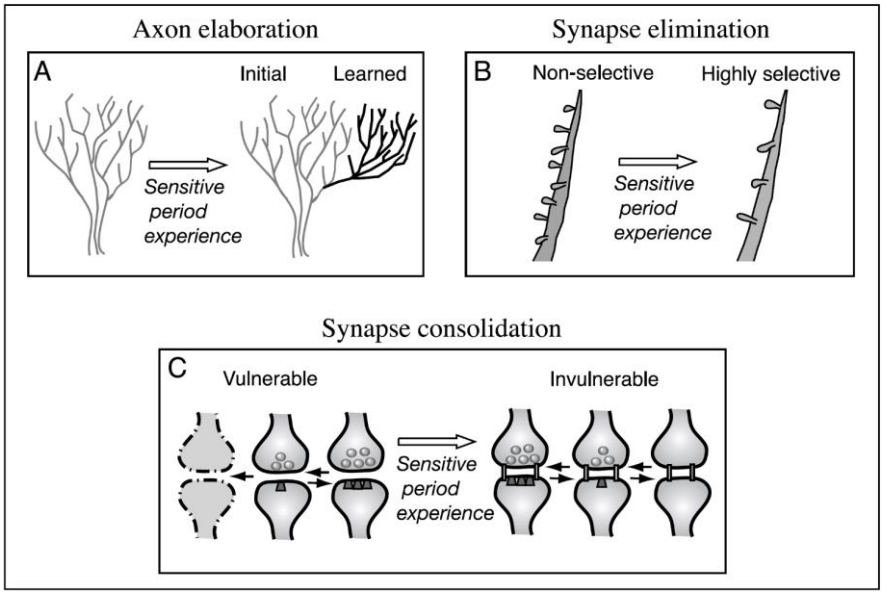


Figure 2. Brain plasticity mechanisms during a sensitive period. Obtained from: Knudsen (2004).

During childhood, the most common source of adversity is the child maltreatment (Hyman et al., 2008). According to the World Health Organization (WHO), child maltreatment is all forms of physical and/or emotional ill-treatment, sexual abuse, neglect or negligent treatment, commercial or other exploitation, resulting in actual or potential harm to the child's health, survival, development or dignity in

the context of a relationship of responsibility, trust or power (WHO, 2006).

The worldwide statistics show that over 1 billion children ages 2 to 17 years have experienced violence in 2015 (Hillis et al., 2016; WHO, 2016). Moreover, epidemiological data showed that at least 64% children in Asia, 56% in Northern America, 50% in Africa, 34% in Latin America, and 12% in Europe, suffer violence (Hillis et al., 2016).

Child maltreatment can be classified in four types: physical abuse, sexual abuse, emotional/psychological abuse, and neglect (WHO, 2006).

1.1.1. Physical abuse

Physical abuse involves intentional physical aggression against a child by an adult, that harm the child's health, survival, development or dignity (Abbasi et al., 2015; WHO, 2006). Examples could be hitting, beating, kicking, shaking, biting, strangling, scalding, burning, poisoning and suffocating (Abbasi et al., 2015; WHO, 2006).

1.1.2. Emotional and psychological abuse

This kind of violence involves the production of psychological and social defects in the growth of a child as a result of the parent or caregiving' behaviour (Abbasi et al., 2015; WHO, 2006). Types of these acts include: the restriction of movement, frightening, yelling, coarse and rude attitude, humiliation, inattention, harsh criticism, denigration, ridicule and other forms of hostile treatment (Abbasi et al., 2015; WHO, 2006). Moreover, this kind of abuse comprises the coercing observation

of violent acts or the incidentally witnessing of violence between two or more persons (WHO, 2016).

1.1.3. Sexual abuse

Sexual abuse is defined as the participation of a child in sexual activity to get physical gratification or financial profit of the person who is obligating the act, who could be an adult or older adolescent (Abbasi et al., 2015; WHO, 2006). Forms of child sexual abuse are asking or pressuring a child to participate in sexual activities, exhibiting the genitals to a child, showing pornography to a child, viewing or physical contact with the child's genitals and producing child pornography (Abbasi et al., 2015).

1.1.4. Neglect

Child neglect is the act or omission of a parent or another caregiver to provide the adequate needs for the development and the well-being of the child (Abbasi et al., 2015; WHO, 2006). The neglect could be divided into two groups: physical neglect and emotional neglect. Physical neglect is the failure to provide the basic physical needs like food, clothing shelter, personal hygiene and medical care (Cohen et al., 2017). Emotional neglect is the inadequate givenness of the emotional needs that a child requires, such as lacking in attention, love and nurture (Cohen et al., 2017).

1.2. Child maltreatment in numbers

According to WHO, there are every year an estimated 41,000 deaths attributed to homicide in children under 15 years old (WHO, 2016) and

around 90% involve parents as perpetrators (Children's Bureau, 2018). However, the most shocking data is that the most common victimizer is the mother (39,4 %), followed by the father with 21.4% (Children's Bureau, 2018).

According to the child help organization, between four or five children die as a result of child violence or neglect (Childhelp, 2020). However, this number of deaths could be underestimated because most of the children deaths are wrongly attributed to falls, burns, drowning or other causes (Abbasi et al., 2015). The WHO reported that 23% child were physical abuse, 36% emotional abuse, 26% sexual abuse and 16% suffer neglect **(Figure 3)** (WHO, 2016).

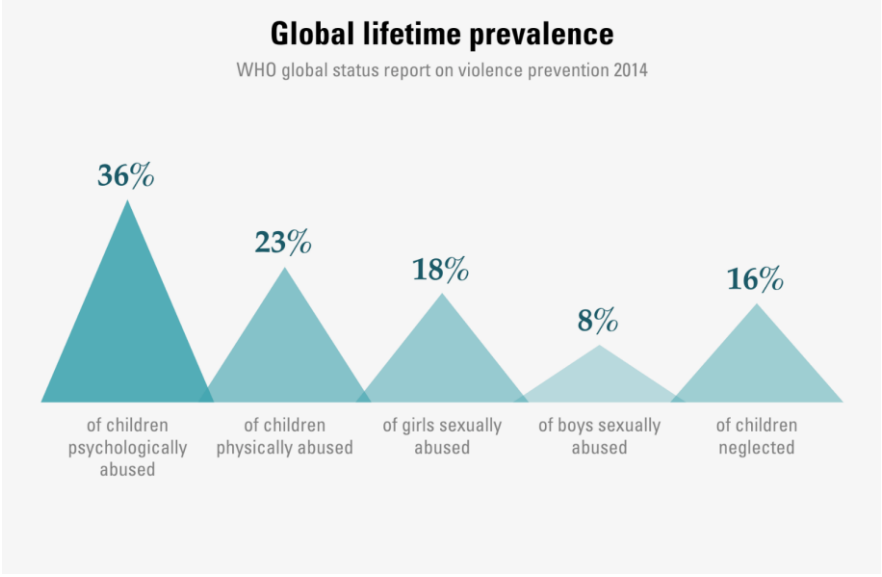


Figure 3. Global prevalence of child maltreatment. Obtained from: Violence Info – Child maltreatment, <http://apps.who.int/violence-info/_embed/global-prevalence-card-of-child-maltreatment>.

Despite these statistics, Cohen (2017) reported that neglect is the most common and silenced type of child maltreatment in many countries, because it is not so evident like the injuries caused by physical abuse or sexual abuse. According to this data, one in five children suffers emotional and/or physical neglect (Cohen et al., 2017). Besides, more than 15% of mistreated children are victims of two or more maltreatment types, and the most common combination is physical abuse and neglect (Children's Bureau, 2018).

1.3. Consequences of child maltreatment

Although child homicides statistics are chilling, deaths represent only a small proportion of the problems caused by child abuse (WHO, 2016, 2006).

Every year, millions of children are victims of non-fatal abuse and neglect (WHO, 2016, 2006). Additionally, child maltreatment also has lifelong consequences (Cohen et al., 2017).

It is known that exposure to child maltreatment increase risk factors to develop other health problems and also raise risk-taking behaviours later in life **(Figure 4)**. Thus, child maltreatment is a crucial factor that contributes to a diverse physical and mental health problems, leading a lot of consequences for the victim, the society, as well as a great economic impact in the public health including costs of hospitalization, mental health treatment, child welfare, and longer-term health costs (Abbasi et al., 2015; Cohen et al., 2017; WHO, 2016, 2006).

Among all the negative consequences that child maltreatment has, stress is one of the principal concern, due to extreme stress can impair the

development of the nervous system (Abbasi et al., 2015). Early-life stress increases the reactivity to future stress but also is associated with cognitive deficits in adulthood (Lupien et al., 2009).

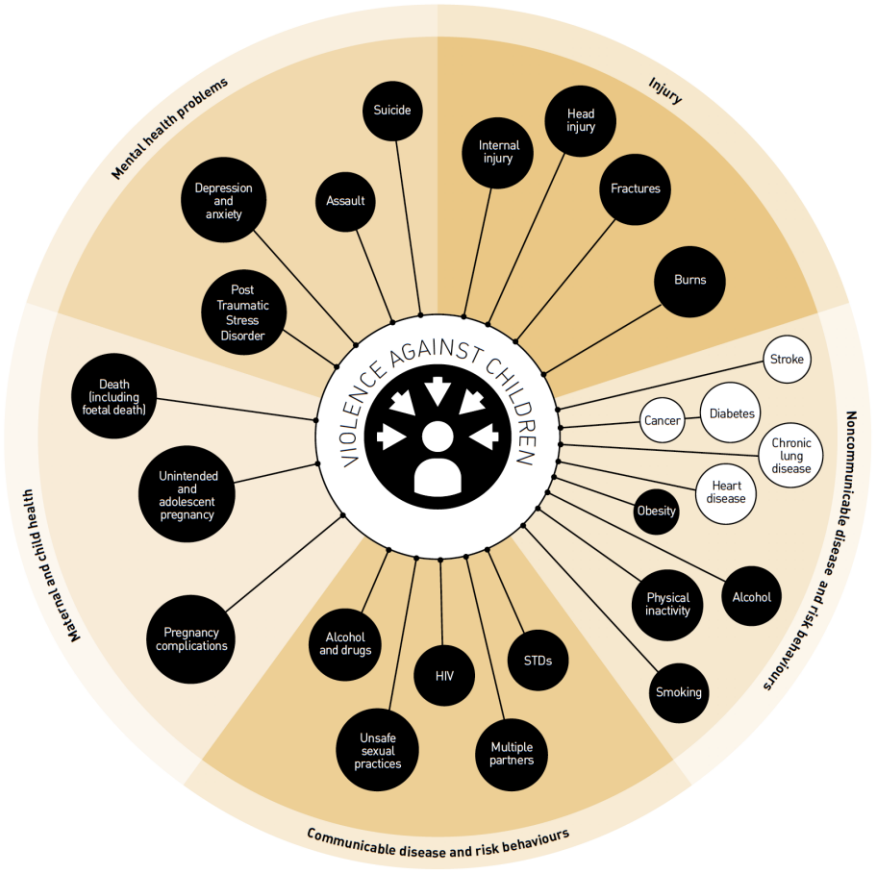


Figure 4. Potential health consequences of child maltreatment. Black circles represent the direct effects; white circles show the indirect effects due to the adoption of high-risk behaviour. Obtained from: WHO (2016).

1.3.1. The stress system

The hypothalamic-pituitary-adrenal (HPA) axis, is the principal neuroendocrine system that regulates stress response and other

functions (Lupien et al., 2009). This axis involves the interaction between the hypothalamus, the pituitary gland and the adrenal gland **(Figure 5)** (Daskalakis et al., 2013; Lupien et al., 2009).

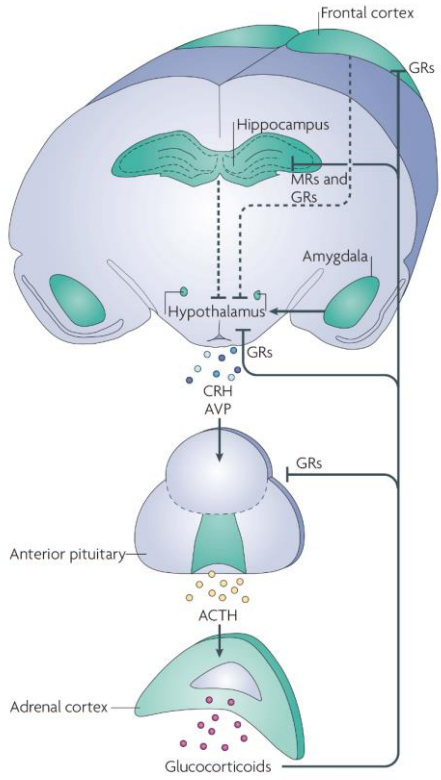


Figure 5. The hypothalamic-pituitary-adrenal axis. Obtained from: Lupien et al. (2009).

The activation of this axis starts with the release of corticotropin-releasing hormone (CRG) and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus (Lupien et al., 2009; Squire, 2008). The CRG and AVP induce liberation of the adrenocorticotropin hormone (ACTH) from the pituitary gland, increasing the synthesis of glucocorticoids in the adrenal cortex (Lupien et al., 2009). These

glucocorticoids bind to the glucocorticoid receptors (GR) and mineralocorticoid receptor (MR) localized in the anterior pituitary, hypothalamus, hippocampus and frontal cortex, acting as a negative feedback. In contrast to these brain areas, the amygdala activates the HPA in order to set in motion the stress response necessary to deal with the threat (Lupien et al., 2009). Moreover, these glucocorticoids act as transcription factors for several genes and alterations in glucocorticoids levels or function, affects the maturation of neurons, myelination, neuronal structure and synapse formation (Lupien et al., 2009).

Clinical and preclinical data have thrown two hypotheses related to stress and brain. The first one is the neurotoxicity hypothesis. According to this hypothesis, chronic exposure to glucocorticoids decreases the capacity of neurons to resist insults, being more vulnerable to be affected by several factors (Lupien et al., 2009). Animal studies showed that increased levels of glucocorticoids stress-stimulate secretion induce adrenal gland enlargement, atrophy of apical dendrites in the hippocampus and also increase the glutamate levels in the hippocampus and the medial prefrontal cortex (mPFC), increasing the vulnerability of the ageing brain to neuronal damage (Lowy et al., 1995). With this hypothesis, the authors conclude that reduced hippocampal size is the result of being exposed for several years to chronic stress or depressive symptoms (Lupien et al., 2009).

The second hypothesis related to early-life stress is the vulnerability hypothesis. This hypothesis also remarks that stress can damage the hippocampus. However, in contrast with the first hypothesis, this proposes that smaller hippocampal volume is a risk factor, in trauma-

experienced individuals, that increase vulnerability to future pathological stress responses (Gilbertson et al., 2002).

Additionally to the alterations observed in the hippocampus, increased amygdala response (van Harmelen et al., 2013), altered amygdala volume (van Harmelen et al., 2013) and reduction in corticolimbic grey matter in the prefrontal cortex (PFC) (Gorka et al., 2014), have been observed in adults with history of childhood maltreatment.

As a consequence of these brain alterations maltreatment-induced, circuits involved in emotional regulation and reward anticipation, have been affected, and several psychiatric disorders could be developed (Teicher et al., 2016). Evidence showed that early-life stress induced by childhood neglect, physical or sexual abuse, are crucial factors that increase the risk for the onset of "internalizing disorders", such as depression and anxiety, and "externalizing disorders", such substance abuse, attention deficit hyperactivity disorder, and delinquency (Charney and Manji, 2004; George et al., 2010; Teicher et al., 2016). Experienced one or more maltreatment-related adverse childhood experiences increase 54% the probability to develop depression (Dube et al., 2003b), 62% for anxiety disorders (Gorka et al., 2014) and 64% for addiction to illicit drugs (Dube et al., 2003a).

Other studies showed that the hippocampus from maltreated individuals who committed suicide showed modifications in the neuron-specific GR but not in non-maltreated individuals who died by suicide (Charney and Manji, 2004). Besides, 80 to 90% of individuals who died from suicide had been diagnosed with severe depression (Charney and Manji, 2004).

1.3.2. The reward system

The principal brain structures that comprise the reward system are the PFC, amygdala, hippocampus, Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc) **(Figure 6)**.

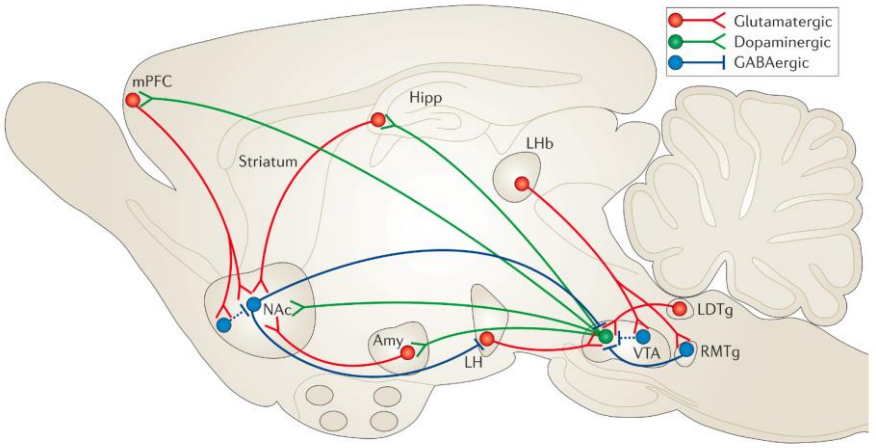


Figure 6. The reward system in the rodent brain. Obtained from: Russo and Nestler (2013).

The main neurons in the VTA are the midbrain dopamine neurons that project to the PFC, central amygdala, basolateral amygdala, hippocampus and the NAc, a part of the ventral striatum (Russo and Nestler, 2013). In the NAc, the principal neurons are the gamma aminobutyric acid (GABA) medium spiny neurons (MSNs), which innervate the VTA by a direct pathway (Dopamine receptors 1 [D1R]-type MSNs) or indirectly via ventral pallidum (Dopamine receptor 2 [D2R]-type MSNs). The NAc also receives glutamatergic projections from the PFC, amygdala and hippocampus. All of these circuitry functions are modulated principally by GABAergic interneurons.

Additionally, the PFC, amygdala and hippocampus, formed glutamatergic connections between them (Russo and Nestler, 2013).

Childhood maltreatment is associated with alteration in blood flow to the PFC, substantia nigra and NAc, reduced striatum volume, attenuated striatal response to reward, as well as changes in neuron trajectory on the NAc (Teicher et al., 2016).

These alterations in the reward system could predispose to suffer depression or develop a substance use disorder (SUD), also known as drug addiction (Teicher et al., 2016; Teicher and Samson, 2016).

2. The neurobiology of addiction

According to the world statistics of drug consumption **(Figure 7)**, there are more than 271 million people who use drugs (UNODC, 2019). Nevertheless, as over 35 million, or almost 13% suffer from drug use disorders (UNODC, 2019). This percentage corresponds to a global prevalence of drug use disorders of 0.71% among the population aged 15-64 (UNODC, 2019). For Europe, the statistics showed that 29% of adults (15-64 years old) have tried illicit drugs during their lives (EMCDDA, 2019).

A drug is a natural or synthetic substance that is designed to produce a specific set of psychological or physiological effects on the human body (Houck and Siegel, 2015). There are a group of drugs produced, legitimacy and prescribed for particular illnesses, injuries, or other medical problems (Houck and Siegel, 2015). However, this kind of drugs could develop pleasurable effects making that people take them for a recreative purpose (Houck and Siegel, 2015).

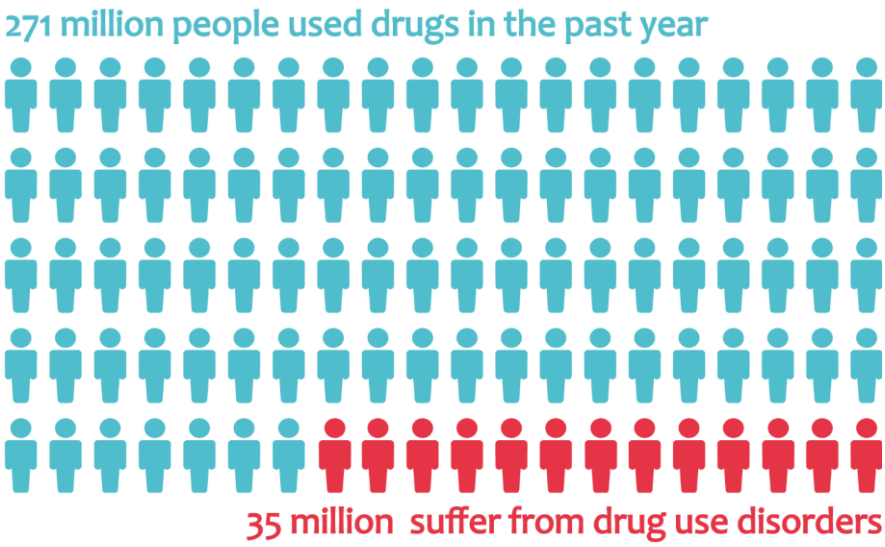


Figure 7. The number for global drug users. Adapted from: UNODC (2016).

Additionally, some substances have no legitimate medical purpose but are produced an ingested entirely for their psychoactive effects (Houck and Siegel, 2015). This group of drugs produced with the exclusive purpose of abuse are called abused drugs, drugs of abuse or illicit drugs (Houck and Siegel, 2015).

Illicit drugs are substances that stimulate or inhibit the central nervous system or also could induce hallucinogenic effects (Uutela, 2001). As a result, these drugs are prohibited under international drug control treaties (Ritchie and Roser, 2018).

The Global Burden of Disease Study 2017 estimated that 166,613 deaths around the world are attributed directly to drug dependency and overdoses; however, other 585,000 globally deaths are caused by illicit drug use as a risk factor (Ritchie and Roser, 2018; UNODC, 2019). The

illicit drugs are classified into four principal groups: opioids, cocaine, amphetamines and cannabis (Ritchie and Roser, 2018).

2.1. Cocaine

Among the illicit drugs, cocaine is the second illicit drug most commonly used after cannabis (UNODC, 2019). According to the WHO, there are 18,1 million people who are cocaine users in 2017, which correspond to 0,4% of the global population aged 15-64 (UNODC, 2019).

As same as the global data, cocaine is the second illicit drug most consumed in Europe with an estimation of 18.1 million cocaine users (12.4 million males and 5.7 million females), being the most commonly used illicit stimulant drug in Europe (EMCDDA, 2019). One of the countries with higher cocaine use is Spain with 2,2% prevalence among adults (15-64 years old) and 2,8% occurrence in young adults **(Figure 8)**, being more used by men than women (EMCDDA, 2019).

Moreover, a study looking for illicit drugs and their metabolites in wastewater to provide data of drug use at a municipal level, showed that some Spanish cities (Barcelona, Castellón, Madrid, Santiago and Valencia) had higher levels of cocaine metabolites in wastewater samples than the levels reported in other European cities participating in the study.

There are many adverse health consequences related with cocaine consumption, however, one of the principals concerns is the developing of cocaine addiction or also known as cocaine use disorder (CUD) (Wagner and Anthony, 2002). The estimation says that around 20% of

the cocaine consumers will shift from cocaine use to CUD (Wagner and Anthony, 2002).

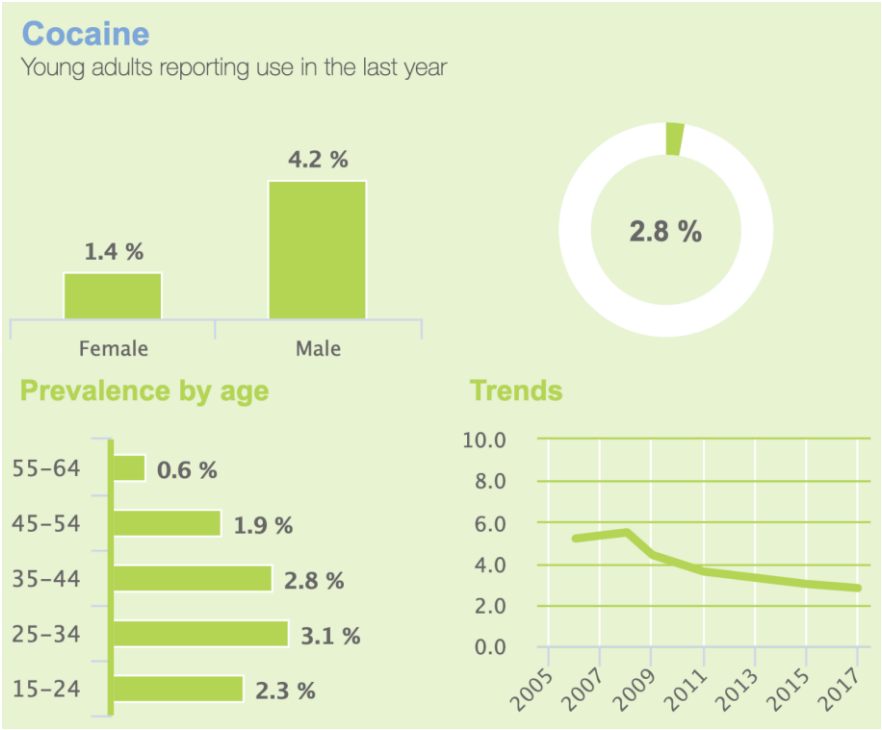


Figure 8. Prevalence of cocaine use during 2017 among young adults in Spain. Obtained from: UNODC (2019).

SUD, including CUD, is a chronically relapsing disorder characterized by (Koob and Le Moal, 2008): the compulsion to seek and take the drug, the loss of control over drug intake, the emergence of a negative emotional when access to the drug is prevented.

2.1.1. Mechanism of action

All the drugs of abuse have in common the induction of dopamine release at the reward brain regions, however, each one has a specific mechanism of action (Volkow et al., 2016).

Cocaine binds to the dopamine, serotonin and norepinephrine transporters, blocking the reuptake of these neurotransmitters into presynaptic neurons (Hummel and Unterwald, 2002; Squire, 2008). As a consequence of the reuptake inhibition, enhanced activity of these neurotransmitters will be evident due to the elevated concentrations of them at the synaptic cleft (Hummel and Unterwald, 2002).

The increased dopaminergic activity at the mesocorticolimbic system directly affects neurological function, creating a conditioned response for use and pleasure, a pattern knowing as cocaine addiction or CUD (Penberthy et al., 2010).

One of the major challenges for CUD-clinical treatment is the high relapse rate (more than 45%) even after long periods of abstinence (D'Ascenzo et al., 2014; Sondheimer and Knackstedt, 2011).

Several studies have indicated that cocaine induces aberrant neuroplastic changes in the mesolimbic system, affecting different neurotransmitter systems including the glutamatergic function (Gass and Olive, 2008); such alterations may be related to CUD and cocaine relapse (Gass and Olive, 2008).

2.1.2. Neuroplastic changes

Increasing consensus suggests that addiction to drugs assumes learning and memory mechanisms normally related to natural rewards, ultimately producing long-lasting neuroadaptations in the mesocorticolimbic system (Stuber et al., 2010).

Several studies evidence that cocaine is capable to trigger the reopening of a "sensitive period" and activate mechanisms juvenile forms of plasticity at molecular, cellular and circuitry levels for altering axonal or dendritic morphologies, making or eliminating synapses, or changing the strengths of synaptic connections (Dong and Nestler, 2014; Knudsen, 2004). These neuroplastic changes are triggered in brain reward regions including the mPFC, the VTA, and the ventral striatum (also known as NAc) (Koob et al., 2014). Therefore, cocaine addiction is basically an acquired behavioural state in vulnerable individuals after repeated exposition to cascades of emotional and motivational experiences (Dong and Nestler, 2014).

Besides, changes in each brain region are associated with the three-stages of drug addiction cycle (Koob et al., 2014; Squire, 2008). These three stages are: binge/intoxication, withdrawal/negative effects, and preoccupation/anticipation **(Figure 9)** (Koob et al., 2014; Volkow et al., 2016).

As mention before, the NAc is a key component of the reward system and is involved in the mediation of addiction-related behaviour (Dong and Nestler, 2014). Clinical and preclinical evidence showed that cocaine induces alterations in the glutamatergic synaptic function at the NAc (D'Ascenzo et al., 2014; Hemby et al., 2005). These accumbal modifications have been related to the drug-seeking behaviour (Hemby et al., 2005).

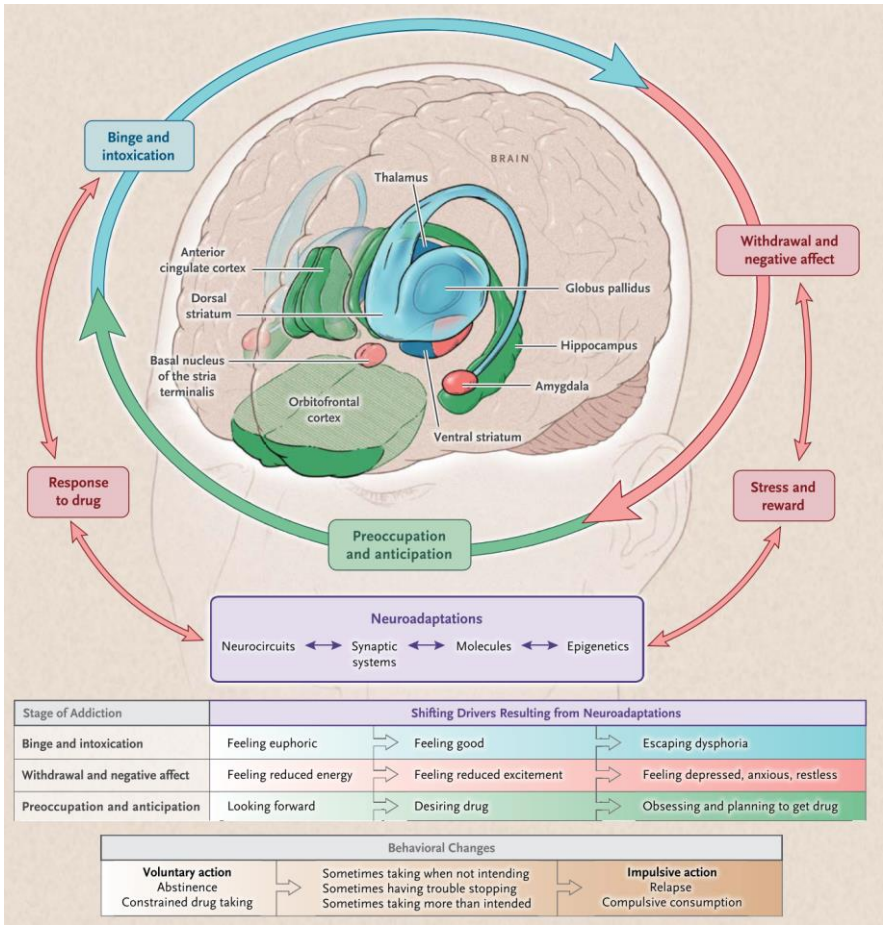


Figure 9. Stages of the addiction cycle. Obtained from: Volkow, Koob and McLellan (2016).

2.1.1. Long-term potentiation

On hand with this, animal models showed that cocaine alters long-term potentiation (LTP) and long-term depression (LTD) at glutamatergic synapses in the NAc (D'Ascenzo et al., 2014). The LTP is a persistent phenomenon that increases synaptic strength that participates in learning and memory in several brain regions (Squire, 2008).

The LTP induction depends on the elevation of calcium (Ca^{2+}) intracellular concentration at pre- and/or postsynaptic neurons (Purves et al., 2004; Squire, 2008), and most of this Ca^{2+}depends on the activation of the ionotropic glutamate receptors (Purves et al., 2004; Squire, 2008).

Among the glutamate receptors, we can mention the N-methyl-D-aspartate (NMDA) and the alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are colocalized at the dendritic spine of the postsynaptic neuron. In standard conditions, only the NMDA receptor is Ca^{2+}permeable. However, if the postsynaptic neuron is at normal resting membrane potential, the NMDA receptor is blocked by physiological concentrations of magnesium (Mg^{2+}) avoiding the entrance of Ca^{2+} (Purves et al., 2004; Squire, 2008).

The activation of AMPA receptors allows the entrance of sodium mediating the depolarization of the membrane. Consequently, the removal of Mg^{2+} from the NMDA receptors is induced and allows the entry of Ca^{2+} to the postsynaptic neuron. This increased Ca^{2+} concentration triggers the LTP induction and the activation of several Ca^{2+} dependent signalling cascades (Purves et al., 2004; Squire, 2008).

The signal transduction cascades involve the activation of phosphatases like the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC) (Purves et al., 2004; Squire, 2008). These enzymes induce the insertion of new AMPA receptors into the postsynaptic membrane, increasing the sensitivity to glutamate (Purves et al., 2004; Squire, 2008). However, these changes are inducing a temporal strengthening of the synapse.

Long-lasting changes of synaptic plasticity require the activation of transcription factors such as cAMP response element-binding (CREB), to activate the expression of other genes that regulate synaptogenesis, survival, growth of new axons, the formation of new dendritic spines and refinement of synaptic connections (Lonze and Ginty, 2002; Merz et al., 2011).

2.2. AMPA-subtype glutamate receptor

As previously exposed, synaptic activity induces the initiation of the LTP, a phenomenon mediated by glutamate receptors. It is known that stimulation of glutamatergic synapses evocates the formation of something called "silent synapses" (Barria, 2009; Kerchner and Nicoll, 2008).

Silent synapses are excitatory glutamatergic synapses whose postsynaptic membrane contains only NMDA receptors, no AMPA receptors (Barria, 2009; Kerchner and Nicoll, 2008). Therefore, although glutamate binds to NMDA receptors, the absence of functional postsynaptic AMPA receptors, keeps the synapse incapable to mediate postsynaptic responses (Barria, 2009; Kerchner and Nicoll, 2008).

In this sense, cocaine has been proposed as an experience capable to induce new silent synapses but also to unsilencing the existing ones, via recruitment of AMPA receptors resulting in postsynaptic strengthening of synaptic transmission (Dong and Nestler, 2014).

Cocaine-induced formation of silent synapses is dependent of CREB and ΔFosB activation **(Figure 10)** (Brown et al., 2011; Russo et al.,

2010). These genes produce the insertion of new NMDA receptors enriched in GluN2B subunit, the type of NMDA receptors related to nascent and immature synapses (Koya et al., 2012).

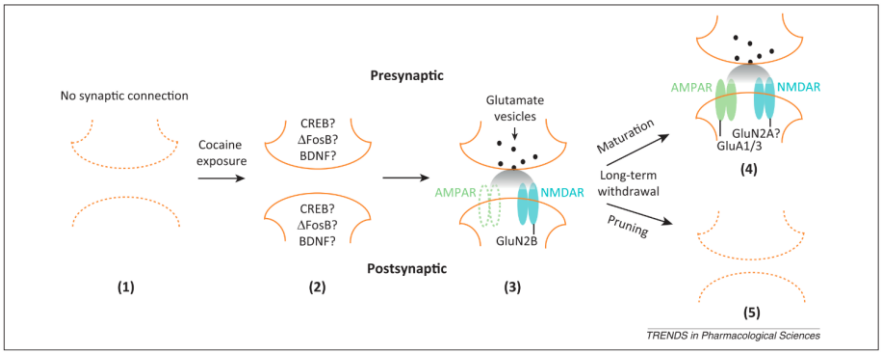


Figure 10. Scheme of a silent synapse-based hypothesis of cocaine-induced synaptic formation following repeated cocaine exposure. Obtained from: Dong and Nestler (2014).

However, cocaine exposure activates cellular cascades which induce the maturation of these synapses, establishing an addicted behaviour (Dong and Nestler, 2014). Besides, long-term cocaine withdrawal could also induce the maturation of these synapses by recruiting an especial type of calcium-permeable AMPA receptors (Lee et al., 2013).

AMPA receptors are made up of four subunit proteins (GluA1, GluA2, GluA3 and GluA4) and are normally assembled by GluA2 in complex with GluA1 or GluA3 (Bowers et al., 2010). However, GluA2-lacking AMPA receptors are calcium-permeable and are capable to induce a higher synaptic strengthening (Bowers et al., 2010).

Animal studies show that extended access to cocaine self-administration (SA) increases the number of GluA2-lacking AMPA receptors in several brain regions, depending on the three stages of cocaine addiction

(Bowers et al., 2010; Conrad et al., 2008; Kalivas, 2009; Pierce and Wolf, 2013), being regulators of behaviours related to this addiction (Goffer et al., 2013; Martínez-Rivera et al., 2017).

2.2.1. Nucleus accumbens

Huang et al. (2009) report increased levels of GluA1 and GluA2 in the NAc of male rats after cocaine exposure, specifically at the shell MSNs. In line with this, extended access to cocaine SA in rats increase the GluA2-lacking AMPA receptors in the NAc, contributing to cocaine-seeking (Conrad et al., 2008; Kalivas, 2009; Pierce and Wolf, 2013).

Moreover, Hemby et al. (2005) reported in cocaine self-administered rhesus monkeys, elevations in both of these subunits (GluA1 and GluA2) in the NAc, and these modifications were proposed as responsible of cocaine reinstatement (Hemby et al., 2005). Human post-mortem studies demonstrated elevations of GluA2 in the NAc of cocaine overdose victims (Hemby et al., 2005). However, no significative changes were observed in GluA1 protein expression (Hemby et al., 2005).

As mention before, CUD-induced neuroplastic changes facilitate cocaine relapse even long abstinence periods. Animal studies showed that extinction training, an inhibitory learning that decreases cocaine-seeking behaviour in the absence of cocaine, evocate alterations in GluA1 and GluA2 AMPA subunits in the NAc of self-administered rats (Choi et al., 2011). Choi et al. (2011) corroborated that increased GluA1 level has a positive relation with the level of extinction achieved during SA, confirming that GluA1 facilitates elimination of cocaine seeking. It

is possible that GluA1 could restore glutamatergic tone in this brain area, decreasing the propensity of cocaine relapse by the restore of the cortical-accumbal tone by the upregulation of AMPA receptor function (Choi et al., 2011).

After prolonged withdrawal, Conrad et al. (2008) observed in rats, an up-regulation of the synaptic GluA2-lacking AMPA receptors in the NAc after cocaine SA (during withdrawal). The higher conductance of these new receptors induced an increased reactivity of accumbal neurons to cocaine-related cues, inducing drug craving and relapse, concluding that these GluA2-lacking AMPA receptors are responsible for the incubation of cocaine craving (Conrad et al., 2008).

As well, increased levels of surface/intracellular ratios of GluA1 and GluA2 were observed in the NAc of cocaine-sensitized rats (Boudreau and Wolf, 2005). There was a positive correlation between the degree of behavioural sensitization to cocaine and the GluA1 and GluA2 surface/intracellular ratios, demonstrating that cocaine sensitization requires redistribution of AMPA receptors in this brain area (Boudreau and Wolf, 2005).

This AMPA receptor redistribution are induced by the activation of D1R as demonstrated by Chao et al. (2002). Chao et al. (2002) observed that the incubation of MSNs and interneurons with SKF 81297 (D1R agonist) stimulates the cell surface of GluA; this effect was reduced by the administration of D1R antagonist SCG 23390 (Chao et al., 2002). Besides, the GluA1 externalization needs the protein kinase A (PKA)-phosphorylation of this subunit (Mangiavacchi and Wolf, 2004). Based on these results, Boudreau and Wolf (2005) propose that AMPA

receptors redistribution is one of the mechanisms responsible for the increased vulnerability of cocaine-seeking reinstatement.

In addition to this observations, it was also reported that microinjections of AMPA into the NAc (shell and core) evocates dose-dependently cocaine-seeking behaviour and also that antisense oligonucleotides against GluA1 mRNA blockade AMPA- and cocaine-primed reinstatement (Ping et al., 2008). This help us to conclude that NAc is fundamental in reinstatement.

In line with this, previous studies have already shown that AMPA receptors antagonists locally administered in discrete brain areas or systemically administered, reduced cocaine intake and cocaine seeking behaviour. Previous studies have shown that infusions of cyanquixaline (CNQX) (AMPA glutamate receptor antagonist) into the NAc decrease levels pressing for cocaine in rats (Suto et al., 2009a), reduce motivation for cocaine after abstinence period and blocked the cocaine reinstatement-induced by intra-PFC cocaine infusion (W.-K. Park et al., 2002). Moreover, this effect of CNQX was selective for cocaine (Doyle et al., 2014).

Other studies have also reported that CNQX modulate cocaine-induced SA (Bäckström and Hyytiä, 2003) or the reinstatement to cocaine seeking (Bäckström and Hyytiä, 2006).

2.2.2. Ventral Tegmental Area

The extensive research about the effects of cocaine in the VTA show that repeated exposure to this psychostimulant induce an elevation of extracellular dopamine, decrease sensitivity of D2 autoreceptors and

elevate levels of tyrosine hydroxylase (TH) (the rate-limiting enzyme in dopamine synthesis) (Churchill et al., 1999).

Additionally, preclinical studies confirmed that cocaine-evoked changes in the AMPA-receptor composition at the VTA potentiates the excitatory transmission and firing of dopamine neurons (Lüscher, 2013a, 2013b), modifying the AMPA receptor composition in this brain area. Moreover, Churchill et al. (1999) showed that repeated cocaine administration yields long-term augmentation in the protein level of GluA1, in the VTA of rats behavioural sensitized to cocaine but no changes in GluA2; this change was evident at 1 day of withdrawal. In line with this, overexpression of GluA1 in the VTA and glutamate release from PFC to VTA induce behavioural sensitization, confirming the importance of glutamate in the rewarding effects of psychostimulants (Carlezon et al., 1997; Schenk and Snow, 1994).

Clinical studies also report an up-regulation of the mRNA and protein level of GluA2 in the VTA of cocaine overdose victims (Tang et al., 2003). SA based animal models have also confirmed the involvement of AMPA receptor in the developmental of addiction. Choi et al. (2011) reported upregulation of GluA1 and GluA2 in the VTA of rats following cocaine SA. They observed that overexpression of GluA1 in this brain area, increased lever-pressing during cocaine SA reflecting an enhanced motivation for the drug (Choi et al., 2011).

Besides, Mahler et al. (2013) showed that microinjections of a CNQX into the VTA reduce reinstatement of cocaine elicited by cues in rats, without affecting cocaine-primed reinstatement. However, the administration of NMDA antagonists did not modify cocaine

reinstatement concluding that VTA AMPA, but not NMDA, transmission is necessary for cocaine relapse (Mahler et al., 2013).

The importance of cocaine-induced changes in AMPA receptor composition in VTA are also implicated in the acquisition of a drug-dependent state, because cocaine exposure is capable to trigger LTP inducing changes on glutamatergic synapses (Malenka and Bear, 2004); this conclude in enhancing AMPA receptor-mediated transmission (Malenka and Bear, 2004). Argilli et al. (2008) demonstrated that a single cocaine injection augmented the GluA1-containing AMPA receptors (GluA2-lacking) at synapses, potentiating AMPA receptors function. Therefore, cocaine generates the switching of AMPA subunits increasing the high conductance of the AMPA receptors. This could be due to the cocaine-induced D1 receptor activation which stimulates the synthesis of GluA1 and other proteins that stabilize the synapsis (Argilli et al., 2008).

In fact, Mameli et al. (2011) showed that cocaine invert the rules of LTP induction in dopamine neurons, replacing GluA2-containing AMPA receptors with GluA2-lacking ones which decrease the NMDA receptor activity. Therefore, the hyperpolarization of the membrane is more efficient due to the changes induced by the previous exposition of the drug (Mameli et al., 2011). Hence, cocaine evoked a specific form of synaptic plasticity based on the GluA2-lacking AMPA receptors (Mameli et al., 2011).

In addition to the implications of AMPA receptor in the acquisition cocaine addiction, its role in relapse has also been seen. Lane et al., (2011) reported that cocaine-induced plasticity in the VTA perseveres

even cocaine is not anymore present. Then, these modifications contribute to cocaine-craving and relapse (Lane et al., 2011). When cocaine is systemically present (30 min after injection), non-TH labelled dendrites (GABAergic neurons) manifest increased GluA1 level (Lane et al., 2011). However, after 72 h, when cocaine is completely depleted from the system, GluA1 augment in TH-containing dendrites and in non-TH dendrites (Lane et al., 2011). This increased GluA1 subunit presence in the GABAergic neurons of the VTA, rise the excitability of these group of neurons leading to a progressive inhibition of VTA dopamine neurons, contributing to the heightened drug-seeking behaviour (Lane et al., 2011). Elevated AMPA receptor activation in GABAergic neurons results in decreased dopamine release in the NAc, meaning that this could be a way to counterbalance the effect induced by cocaine (Lane et al., 2011). In line with this hypothesis, the activation of the VTA GABA receptors triggers the function of the metabotropic glutamate receptor (mGluR) in order to reduce the number of GluA2-lacking calcium-permeable AMPA receptors (CP-AMPARs) (Kelly et al., 2009). This inhibition of VTA dopamine could explain the anhedonia and withdrawal experienced at the absence of cocaine (Miczek et al., 2008).

A study evaluating the vulnerability to cocaine SA show that rats with a high locomotor response to a novel environment (HRs) showed greater probability to cocaine SA than low responders to the novel context (LRs) (Marinelli and White, 2000). This increased vulnerability to cocaine SA could underlie in VTA dopamine neurons due to HRs showed elevated impulse activity in these group of neurons (firing rate and bursting activity) compared with the LRs rats (Marinelli and White,

2000). Moreover, the HRs evidenced significant difference in the dopamine D2 autoreceptor sensitivity, attributable to differences in excitatory glutamatergic inputs (Marinelli and White, 2000).

2.2.3. Prefrontal cortex

The PFC is a brain region involved in several cognitive functions like attention, prioritizing the significance of stimuli, monitoring the temporal sequence of stimuli, referencing stimuli to internal representations or cues, devising abstract concepts and inhibitory control (Jentsch et al., 1999; Narayanan and Laubach, 2017). Another function in which this brain area is involved is in the reward response, due to the modulation of the GABAergic function in the NAc and the VTA dopamine neurons (Lane et al., 2011).

Clinical studies reported an association of the activation of this brain area and cocaine desire. Kilts et al. (2001) observed in cocaine-dependent subjects an increased metabolic activity in the PFC that correlates with cocaine craving (Kilts et al., 2001). In agreement with these observations, studies in self-administered rats showed that cocaine-induced reinstatement is dependent on the elevation of glutamate but no dopamine in the NAc (McFarland et al., 2003). Moreover, this glutamate that blocks the cocaine reinstatement, arrives from the glutamatergic afferents from the PFC, inactivating the AMPA glutamate receptor in the NAc (McFarland et al., 2003).

Likewise, animal studies showed that GluA1-containing receptors elevation after cocaine exposure on dopaminergic VTA neurons (Gao and Wolf, 2007) and accumbal GABAergic neurons (Boudreau and

Wolf, 2005) is dependent of the glutamatergic transmission from the PFC. This GluA1 elevation is trigger by the activation of D1R, a PKA-dependent mechanism, facilitating the LTP (Sun et al., 2005). Then, after cocaine exposure, increased GluA1 levels in GABAergic neurons in the VTA will induce a higher inhibition of VTA dopamine neurons (Lane et al., 2011), explaining anhedonia and withdrawal associated to cocaine abstinence. Therefore, studies are in agreement that glutamatergic projections from the PFC to the NAc is critical for the reinstatement of cocaine-seeking behaviour (Bowers et al., 2010).

2.3. cAMP response element-binding

As previously exposed, cocaine exposure induces LTP and this process requires new protein synthesis (Argilli et al., 2008; Heshmati, 2009). It was observed that gene expression after short-term cocaine exposure, is mostly dependent on CREB, and gene expression after long-term cocaine treatment is more dependent on ΔFosB (McClung and Nestler, 2003).

CREB is a transcription factor that, once phosphorylated, promotes transcription of cAMP response element-regulated genes (De Rasmo et al., 2009). CREB phosphorylation is mediated by the cAMP-dependent PKA, a molecular element that its formation is stimulated by chronic cocaine use (Carlezon et al., 1998).

Studies showed that CREB overexpression in the NAc decreases the rewarding effects of cocaine and makes ineffective doses of this drug aversive (Carlezon et al., 1998). In line with this, Barrot et al. (2002)

reported that sustained elevations of CREB activity in the NAc produce an anhedonia-like profile.

This activation of CREB has been proposed as one neuroadaptation to compensate cocaine-induced dopamine release in the GABAergic neurons of the NAc, because CREB induces the expression of dynorphin **(Figure 11)** (Muschamp and Carlezon, 2013). In fact, a study in humans showed that cocaine users display the elevation of dynorphin mRNA and kappa-opioid receptor (KOR) in the NAc (Hurd and Herkenham, 1993). Dynorphin is an opioid peptide that acts at KORs and mediates GABA synaptic transmission (Roberto and Gilpin, 2014) and decreases dopamine release from VTA dopamine neurons to NAc (Nestler, 2004).

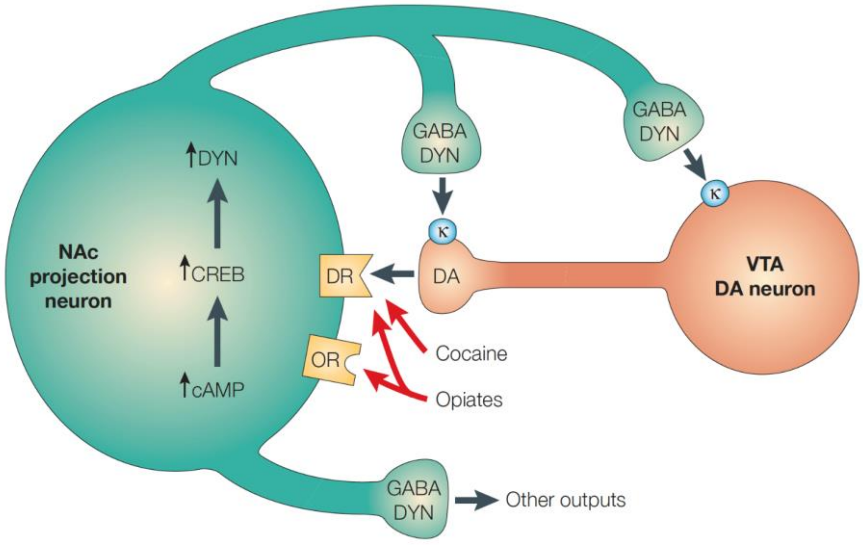


Figure 11. Regulation of CREB and dynorphin by cocaine. Obtained from: Nestler (2001).

Therefore, CREB-mediated dynorphin augmentation due to cocaine exposure enhances KOR activity and reduces dopamine transmission in VTA and NAc (Muschamp and Carlezon, 2013), antagonizing the negative effect of cocaine (Carlezon et al., 1998). However, dynorphin elevation-dependent of continuous cocaine exposure could inhibit the basal dopamine release from the VTA to the NAc (Carlezon et al., 1998). Therefore, this mechanism contributes to the sensitization to the reward-related properties of cocaine but also to the dysphoria seen during withdrawal (Carlezon et al., 1998; Nestler, 2001).

Animal studies showed that repeated cocaine administration in mice decreases CREB expression (Riday et al., 2012) but increases the phosphorylated form of CREB (pCREB) (Mattson et al., 2005) in the NAc, which attenuates the rewarding effect of the drug.

Moreover, a decreased expression of CREB in this same brain area reduces cocaine reinforcement and facilitates the extinction of cocaine-seeking behaviour in a self-administered rats (Larson et al., 2011).

Regarding the VTA, Tang et al. (2003) observed in cocaine overdose victims, a significant elevation of CREB protein level in the VTA. In accord with this observation, preclinical data showed that cocaine priming induced the phosphorylation of CREB in the VTA of mice, inducing reinstatement to conditioned place preference (CPP) (Kreibich and Blendy, 2004).

Other CREB target genes are GluA1 (Olson et al., 2005) and TH (Nestler, 2001; Olson et al., 2005). Overexpression of CREB in the VTA showed an elevation of both of these genes, raising the possibility

that CREB may exert its effects on drug reward, via regulation of these genes (Olson et al., 2005).

In sum, upregulation of CREB after cocaine administration represents a mechanism of "motivational tolerance and dependence" making that the removal of the drug left in the subjects an amotivational and depressed-like state (Nestler, 2004), a topic that will be discussed later.

3. Vulnerability factors to develop drug addiction

Between subjects, there are some factors that differentiate the individuals that are more prone for addiction. Some of the multifactional causes for a vulnerable phenotype are: genetic, personality traits, experience of trauma or abuse, sociocultural influences or various comorbidities and epidemiological observations (Le Moal, 2009; Volkow et al., 2019).

3.1. The telescoping effect

According to the European Drug Report, experience of drug is more frequently in males than in females (57.8 million and 38.3 million respectively) (EMCDDA, 2019). Moreover, men are more prone to psychostimulant use than women (UNODC, 2018). However, in women, substance use tends to progress faster than in males, a phenomenon known as telescoping (Haas and Peters, 2000; UNODC, 2019).

The telescoping effect is especially evident in the case of psychostimulant such as cocaine for which women show higher rates of cocaine use, earlier age of onset and maintain abstinence for a shorter

time-period than men (Johnson et al., 2019; Swalve et al., 2016; Zlebnik, 2019).

Moreover, the studies show the presence of sex differences in all the phases of drug addiction, in all stages of drug addiction **(Table 1)** (Becker et al., 2017). Therefore, it must be biological differences between both sexes that impact the how each responds to drugs and participate in compulsive behaviours like addiction (Becker et al., 2017).

Epidemiologic studies showed that women use cocaine in higher quantity and frequency than men, report more symptoms at low doses of cocaine and showed a higher percentage of cocaine dependency (Chen and Kandel, 2002).

Besides, a study evaluating differences in crack cocaine users found that women had an increased severity of cocaine use than men (Sanvicente-Vieira et al., 2019). In line with this, Haas and Peters (2000) reported that female cocaine abusers exhibit a shorter latency from the first use to abuse and more admissions to treatments than men, demonstrating that women evolve differently in terms of cocaine addiction (Haas and Peters, 2000).

Structural neuroimaging observations report that females may be more susceptible to the negative effects of chronic cocaine on brain volume and also greater neural activation to cocaine cues relative to men (McHugh et al., 2018).

Table 1. Sex differences in stages of addiction. Obtained from: Becker, McClellan and Reed (2017).

	Men	Women
Acquisition	Initial exposure to drug food or activity. Take drugs and engage in risky behaviour to be part of the group more than women do.	Initial exposure to drug, food or activity. May experience more pleasurable response to drugs than men. More likely to self-medicate than men.
Escalation	Slower escalation than women.	Increase in amount and frequency of drug taking. For those at risk for addiction, escalation is more rapid than for men.
Maintenance	The addictive behaviour is established and stabilizes. Males stabilize at lower doses of drug than do females.	The addictive behaviour is established and stabilizes. Females stabilize at higher doses of drug than do males. Side effects of drugs are greater for women.
Withdrawal	Men exhibit greater symptoms of withdrawal from alcohol than women.	Female smokers report increased negative affect during withdrawal and experience a greater stress response than men do.
Relapse	Men have longer periods of abstinence than women.	Women are more likely to relapse than men and do so more sporadically.

Animal studies have also demonstrated sex differences in drug consumption. In SA experiments, have been observed that female rats acquire cocaine SA behaviour at a quicker rate and higher percentage (Lynch, 2008), performing a greater number of infusions and consuming more cocaine than males (Cummings et al., 2011; Davis et al., 2008; Johnson et al., 2019; Peterson et al., 2014). Moreover, female rats show more incentive motivation for cocaine than males (Algallal et al., 2019), obtain more cocaine infusions during SA progressive-ratio schedule (Lynch and Taylor, 2004), self-administered more cocaine (Lynch and Taylor, 2004) and develop psychomotor sensitization to cocaine (Hu and Becker, 2003; Van Haaren and Meyer, 1991).

Nevertheless, other studies have reported different results. Whilst Caine et al. (2004) appreciated no between-sex differences, Swalve et al. (2016) reported that male rats acquire at a higher percentage and in fewer sessions but once acquisition criteria were met, females did consume more cocaine.

Regarding cocaine relapse, Doncheck et al. (2020) reported in female rats, a leftward shift in the dose-response curve for cocaine reinstatement, being more susceptible to cocaine-primed reinstatement than males.

Together, animal and human studies provide compelling evidence of fundamental differences in the motivational properties of cocaine between males and females.

3.2. Comorbidity between addiction and depression

Comorbidity refers to the co-occurrence of one disorder that could increase the likelihood of another to occur (Rappeneau and Bérod, 2017; Torrens et al., 2015). In this case, we want to focus on the comorbidity of substance use and another mental disorder such as depression.

Regarding mental disorders, the most prevalent comorbidity is depression and SUD, with numbers of affected up to 80% (Torrens et al., 2015). The relevance of this comorbidity is that individuals who suffer both disorders are in high risk of emergency admissions, increased rates of psychiatric hospitalisations and a higher prevalence of suicide (Torrens et al., 2015). The diagnosis of this comorbidity is critical because could improve considerably the treatment, clinical evolution, medication adherence and recovery of these patients (Rappeneau and Bérod, 2017).

Major depressive disorder (MDD) is the most common psychiatric disorder affecting more than 264 million people worldwide (García-Marchena et al., 2019; GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). This disorder is characterized by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness, and poor concentration (Matthews and Robbins, 2003; Nestler et al., 2002; Rappeneau and Bérod, 2017; WHO, 2017). Moreover, these core symptoms could finish in suicidal ideation (Patel et al., 2016).

Depression will affect one out of every five people in their lifetime and is the leading cause of disability worldwide (Ménard et al., 2016). Besides, depression is almost twice as common in women than men **(Figure 12)** (Nestler et al., 2002).

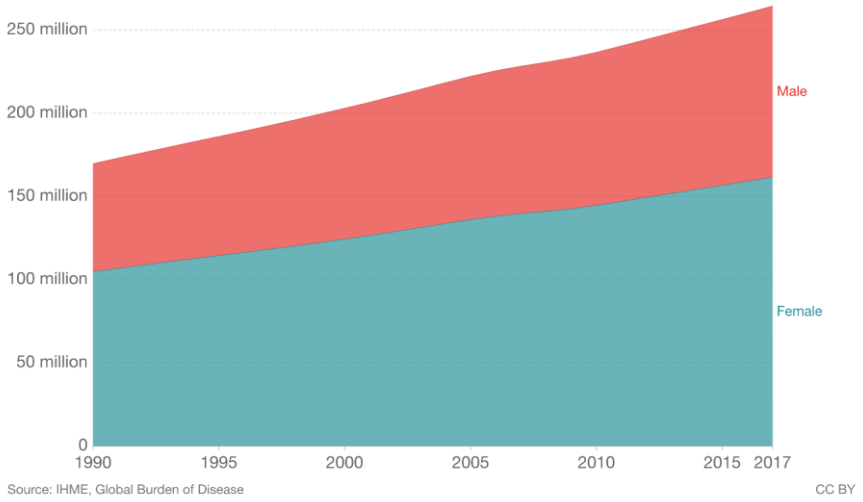


Figure 12. Global overview of the prevalence of depression. Obtained from: https://ourworldindata.org/grapher/number-of-people-with-depression.

Clinical evidence yields that subjects with major depression have increased vulnerability to developing SUD but in other cases, SUD trigger and potentiate the risk of developing major depression during their life than the general population (Torrens et al., 2015). The prevalence rate of SUD in depressive patients is almost twice than the general population (Nestler et al., 2002). On other hand, depression is 3-4 times more prevalent in subjects diagnosed with SUD (Lai et al., 2015). Therefore, these comorbid disorders interact to make each other worse.

Generally, there is an inaccurate diagnosis of this comorbidity, because it is difficult to distinguished which disorder was first, but also because chronic depression symptoms could be attributed to the drug use (García-Marchena et al., 2019). Substance use and depression are interlinked in different ways, which are in sometimes overlapping (Torrens et al., 2015). There are three possibilities of the triggering of this comorbidity: chance, selection bias or causal association (Torrens et al., 2015).

"Chance" refers that the combination of depression and substance use represent two or more independent conditions. In this case, addiction is a behavioural disorder (i.e. compulsive loss of control, at times uncontrollable craving, seeking and use despite devastating consequences) with several additive objects (substances, gambling, sex) occurring in predisposed individuals in which a trait (impulsivity) determines the neuroplasticity induced by psychoactive substances (Swendsen and Le Moal, 2011).

"Selection bias" means that depression is the risk factor to substance use disorder (Rappeneau and Bérod, 2017; Torrens et al., 2015). In other words, the individual trend to use drugs in order to deal with problems associated with depression (Rappeneau and Bérod, 2017; Torrens et al., 2015).

"Causal association" this occurs when the SUD trigger the development of depression (Rappeneau and Bérod, 2017; Torrens et al., 2015). This is also known as substance-induced disorder. In this case, depression could be a long-term disorder or just temporal condition, produced as

a consequence of intoxication or withdrawal conditions (Rappeneau and Bérod, 2017; Torrens et al., 2015).

Another possibility is the presence of some factors that increase the likelihood of depression and substance use disorder explaining their association, like exposure to early-life adversity (child maltreatment) (Rappeneau and Bérod, 2017; UNODC, 2018).

3.2.1. Molecular mechanism of depression

As previously described, the mesolimbic dopamine system is associated with the rewarding effects of food, sex and drug of abuse (Nestler, 2005), being this brain system the molecular pathway that MDD and SUD shared in common (Nestler and Carlezon, 2006).

Many studies reports in post-mortem analysis and neuroimaging evaluations of depressed patients, reductions in grey-matter volume in the PFC and hippocampus, brain structures that mediate cognitive aspects of depression, such as feelings of worthlessness and guilt (Krishnan and Nestler, 2008).

Besides, some neurotrophic factors in brain reward areas are involved in the aetiology of depression function (Nestler et al., 2002). Regarding the CREB function in the brain cortex, a study in patients with depression showed a decreased CREB level in the temporal cortex which was reversed with antidepressant treatment (Dowlatshahi et al., 1998).

Other studies have demonstrated that antidepressant administration increases expression of CREB mRNA and protein levels in the

hippocampus (D'Sa and Duman, 2002). In agreement with this observation, Krishnan and Nestler (2008) showed that antidepressants administration upregulates the expression of CREB, due to the stimulation of serotoninergic dependent G-protein-coupled receptors (Krishnan and Nestler, 2008). Meanwhile, Chen et al. (2001) demonstrated that CREB overexpression in hippocampus, induce the expression of brain derived neurotrophic factor (BDNF), evocating an antidepressant effect (Chen et al., 2001). The hypothesis suggested is that BDNF levels induced by antidepressants may improve BDNF-mediated signalling in the hippocampus, improving the function of this brain area (Chen et al., 2001).

Additionally, the upregulation of CREB activity in the NAc induced by stress, caused a pro-depressive phenotype in mice (Nestler, 2015). The increased CREB activity induces the expression of BDNF and dynorphin, two genes that contribute to depressive-related behaviours in the NAc (Nestler, 2015). In fact, dynorphin activates KORs in VTA neurons, inhibiting dopaminergic activity and contributing to anhedonia and the development of depressive symptoms (Nestler, 2015).

Recently, GluA2-lacking AMPA receptors have been suggested as regulators of depression-like behaviour (Goffer et al., 2013), but also has been proposed that modifications in GluA2-lacking AMPA transmission in the VTA and NAc as a common target linking cocaine addiction and mood disorders (Martínez-Rivera et al., 2017).

Sub-anaesthetic dose of ketamine, a NMDA receptor blocker, induce a rapid antidepressant effect (Cryan and O'Leary, 2010), also observed in depressed patients (Skolnick, 2008).

A possible mechanisms that can explain this ketamine-induced antidepressive effect is because of the activation of the mammalian target of rapamycin (mTOR), a ubiquitous protein kinase involved in protein synthesis, formation of dendritic spines and other synaptic activity in the PFC (Cryan and O'Leary, 2010). Ketamine-induced mTOR promote the translation of GluA1 at the postsynaptic site, potentiating the AMPA receptor activity (Doan et al., 2015).

The chronic administration of maprotiline, another antidepressant, also up-regulates GluA1 protein expression in the NAc and hippocampus of mice which leads increased calcium permeability, increasing synaptic function in these brain areas (Tan et al., 2006).

Moreover, fluoxetine triggers too the augmentation of AMPA activity inducing the phosphorylation of GluA1 in the PFC, hippocampus and striatum (Svenningsson et al., 2002); in contrast, serotonin depletion in the PFC decreases the expression of GluA1 but increase GluA2 in the cerebral cortex (Shutoh et al., 2000).

In line with this, Duric et al. (2013) observed in post mortem hippocampus of subjects with MDD, a down regulation of the mRNA GluA1 level (encoded by the Gria1 gene). Moreover, the same observation was detected in the hippocampus of rats with depression-like behaviour (Duric et al., 2013).

In another stress model of depression was observed a GluA1 reduction in the PFC of rats, due to the degradation by the ubiquitin-proteasome pathways (Tan et al., 2006). This loss of glutamate receptor could explain the decreased glutamatergic activity that leads to the deficit of

PFC-mediated cognitive processes existing in depression (Tan et al., 2006).

In the NAc, decreased concentrations of GluA2-containing AMPA receptors are also associated with depressive states (Doan et al., 2015).

According with the previous data, GluA1 knockout mice showed a higher vulnerability to depression, being used as a model to investigate the physiopathology underlying the depressive states (Chourbaji et al., 2008).

Although the information presented, the biological mechanism that underlie the association between MDD and SUD remain largely unknown, being necessary to explore deeply in order to improve the life quality of the patients living with this comorbidity (Rappeneau and Bérod, 2017).

3.2.2. Trait impulsivity and depression

Among the dopamine behavioural dependent processes, we can find two extremes, depression and impulsivity. We previously described that hedonic processing and motivation depends closely on dopamine, therefore, depression and impulse control disorders (like SUD), depends on the modulation of this neurotransmitter.

By definition, impulsivity is a major personality and temperament dimension consisting of maladaptive behaviour and characterized by poorly conceived, prematurely expressed, unduly risky, or inappropriate actions often resulting in undesirable consequences (Dalley et al., 2011).

Impulsivity could be divided in two major processes and each one depends of different neural network: cognitive impulsivity and motor impulsivity (Houeto et al., 2016). Cognitive impulsivity is the failure to tolerate delays of reinforcements, preferring immediate smaller rewards over distant larger ones (Dalley and Ersche, 2019). Motor impulsivity implies the inability to withhold, stop, or postpone a response, developing anticipatory actions (Dalley and Ersche, 2019; Dent and Isles, 2014; Granö et al., 2007).

Clinical studies reported increased impulsivity in adults with depression (Peluso et al., 2007) or later MDD diagnosis (Granö et al., 2007), suggesting impulsivity as a predictor of the development of major depression (Granö et al., 2007). Also, Corruble et al. (2003) described three characteristics of impulsivity in adults with severe depression: behavioural loss of control, non-planning activities and cognitive impulsivity.

Additionally, depression and impulsivity increases suicide attempt as demonstrated by (McHugh et al., 2019). Also, Wang et al. (2015) showed that MDD patients with higher impulsivity were more prone to have suicidal ideations even when depression had been ameliorated.

In line with these studies, some authors have evaluated the association between impulsivity and childhood adversity in depressed adults, concluding that patients with history of childhood trauma showed higher impulsivity and higher suicidal behaviour than the others without childhood adversity (Brodsky et al., 2001).

Additionally to depression, impulsivity is also associated with other behavioural traits like novelty seeking (Dalley et al., 2011). Therefore, impulsivity is a trait that can increase the predisposition to suffer drug addiction (Adams et al., 2019; Butelman et al., 2019; Jupp et al., 2020; Nicholls et al., 2014; Rømer Thomsen et al., 2018).

Because of this reason, impulsivity has been proposed as a endophenotype to investigate the underlying neurobiological mechanisms for many of impulse control disorders including SUD (Dalley and Ersche, 2019).

Clinical studies show that cocaine addiction includes poor inhibitory control for the goal-directed behaviour in the frontal cortical regions, which induce craving for the drug (Barrós-Loscertales et al., 2020; Squire, 2008).

In the four-choice serial reaction time task, a behavioural test to assess waiting impulsivity, study performed in cocaine-addicted patients showed a trend to respond more prematurely or incorrectly than the healthy group (Clark et al., 2006; Dalley and Ersche, 2019). Besides, the cocaine group made disadvantageous decisions because they are less likely to choose the most probable option (Clark et al., 2006; Dalley and Ersche, 2019).

Moreover, resilient cocaine users (recreational cocaine users that never transit to addiction) reported normal levels of impulsivity and no changes in brain morphology normally associated with chronic cocaine use (Morein-Zamir et al., 2015). An interesting observation is that these recreational users did not have history of severe adverse experiences

during childhood (Morein-Zamir et al., 2015). As exposed, early childhood adversity is related with immature prefrontal-limbic connectivity which contributes to increased impulsivity and higher probability to SUD (Volkow et al., 2019)

Preclinical studies also demonstrated that increased impulsivity predicts the predisposition to evolves from drug use to drug abuse (Jupp et al., 2020). In a SA paradigm, rats with high impulsivity met the acquisition criterion faster, in greater percentage and also consumed more cocaine than rats with low impulsivity (Perry et al., 2005). Moreover, in male rats were observed that high impulsivity goes before of the development of compulsive cocaine use (Belin et al., 2008) while in females rats, impulsivity could predict the acquisition of cocaine SA (Perry et al., 2005).

A genetic animal model of impulsivity, the Roman high-avoidance rats showed increased cocaine sensitization (Giorgi et al., 2005), higher number of responses in the SA paradigm (Fattore et al., 2009) and higher vulnerability to self-administer cocaine (Fattore et al., 2009). All of these behavioural alterations were associated with decreased volume and function of some mesocorticolimbic areas related with the development and persistence of cocaine addiction (Fattore et al., 2009; Giorgi et al., 2019; Río-Álamos et al., 2019).

Regarding sex differences in impulsivity, evidence showed that both women and female rats express higher impulsive actions than males (Weafer and de Wit, 2014).

Anker et al. (2008) reported greater impulsive action for cocaine seeking in cocaine self-administering rats; on other wise, Perry et al. (2008) indicates that impulsive choice is a better predictor for cocaine reinstatement in female rats than in males. These results evidenced that women are more sensitive to the chronic effects of cocaine on impulsive action.

Although the molecular substrates of impulsivity and SUD is not fully understood, abnormal activity in the mesolimbic system has been reported in impulsive subjects, suggesting an increase dopamine sensitivity in the VTA (Dalley and Roiser, 2012). Moreover, NAc and other nucleus in the ventral striatum, incorporates cortical and limbic inputs to modulate the inhibitory control of reward-related behaviours throughout D2R (Dalley and Roiser, 2012). Then, elevated D2R activation-cocaine induced could inhibit the inhibitory response control by the PFC (Dalley and Roiser, 2012).

Concerning glutamate, the VTA has an important role in goal-directed behaviour, including drug-seeking behaviour and this process is dependent of the glutamatergic inputs from the PFC (Heshmati, 2009). Once again, AMPA function alterations were associated with impulsivity but also with this personality trait and cocaine use. Nakamura et al. (2000) showed that the administration of NBQX (disodium salt hydrate), a selective competitive AMPAr antagonist, decreased the impulsivity in a dose-dependently way in rats. On other hand, Barkus et al. (2012) reported in a GluA1 knock out mice, an impulsivity phenotype, faster acquisition in the food SA paradigm and decreased capacity to extinguish the SA behaviour.

3.3. Early-life adversity

As exposed in the first chapter, early-life stress during prenatal and postnatal periods, is associated with negative consequences, including maladaptive long-lasting brain effects (Ducci et al., 2009; Hyman et al., 2008). Being victim of abuse or negligence showed an increase risk for developing psychiatric disorders (Lippard and Nemeroff, 2020). For example, history of childhood adversity increase in 28.8% the possibility of depression, 16.5% the risk of illicit drug use (Anda et al., 2006). Human studies showed that individuals that had experienced of early life adversity showed several functional alterations in the reward circuitry like decreased activity in the basal ganglia and attenuation of the ventral striatum activation (Birnie et al., 2020)

Related to illicit drugs, it has been observed that 20% of cocaine users become addicts (Wagner and Anthony, 2002), but the likelihood of shifting from cocaine use to CUD increases two folds in individuals with a history of childhood maltreatment (Ducci et al., 2009). Other studies showed that childhood maltreatment negatively impacts cocaine relapse (Hyman et al., 2008). In general population, the cocaine relapse rate is approximately 45% (Back et al., 2010), however, individuals who have suffered childhood maltreatment, the probability to relapse increases rising 90% (Hyman et al., 2008).

Although clinical studies help to understand the consequences of early-life adversity and cocaine addiction in brain function or structure, animal studies are necessary in order to investigate the underlying molecular and cellular mechanisms behind the cognitive and emotional alterations in individuals exposed to early-life adversity (Krugers et al.,

2017). Moreover, animal models allow to identify the neural targets, critical period modulators, why not everyone who is exposed regularly to a drug becomes addicted and therapeutic solutions for adverse experiences in humans (Nelson and Gabard-Durnam, 2020; Volkow et al., 2019).

4. Animal models of early-life adversity

In the field of life science, at least 59% of the animal studies employs mouse models to investigate the implications on human health, because almost 99% of mouse genes resemble the human genome (Dutta and Sengupta, 2016).

Moreover, the observations in rodent experimental findings in early-life stress models and results from maltreated individuals showed a strong parallelism (Teicher et al., 2016). For example, individuals with history of maltreatment who committed suicide, showed a epigenetic modifications of the neuron-specific glucocorticoid receptor gene in the hippocampus as well as rodents exposed to early-life stress (Suderman et al., 2012).

Therefore, mice give comparable results to humans, being really useful to identified molecular changes and epigenetic mechanisms associated with early-life stress (Schmidt, 2010; Teicher et al., 2016).

4.1. Relation between mice age and human age

At this point, it is necessary to understand the equivalences in mouse age and human being age **(Figure 13)**.The mean mouse lifespan is 18-24 months while the average life expectancy of a human being is 80 years (Dutta and Sengupta, 2016; Mulder et al., 2018).

If we take in count the entire lifespan of both, 9 mouse days is equivalent to 1 human year, however, depending on the developmental stage, this comparison varies (Dutta and Sengupta, 2016; Mulder et al., 2018). At the early life, mice have a shorter and accelerated life in comparison with the human being.

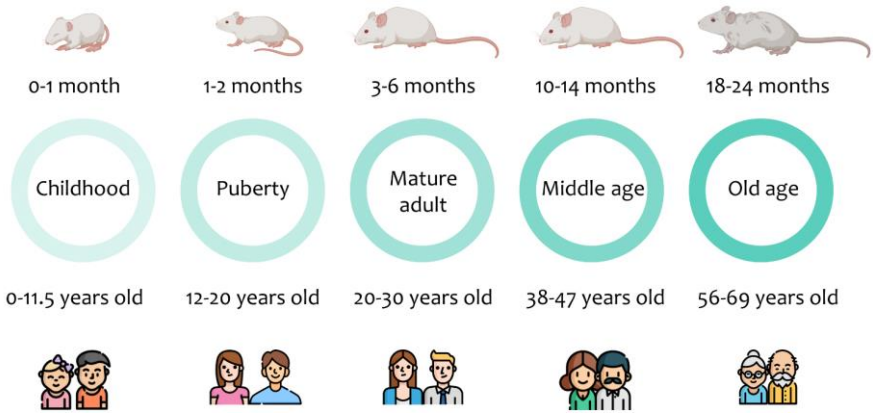


Figure 13. Approximate mouse/human age comparisons. From birth to 1 month, mice develop 150 times faster than human beings; 1-6 months, mice mature 45 times faster: more than 6 months, mice age 25 times faster. Adapted from: The jackson laboratory (2017), figure V.3.

4.1.1. Childhood

During the first month, a mouse matures 150 times faster than a human being, therefore a mouse of 28 days is comparable to a child of 11.5 years of age (Mulder et al., 2018).

The transition from childhood to adulthood, the puberty, involves the maturation peak of the HPA axis, which is characterized by alteration in gonadotropin levels in circulation and elevated levels of sex steroids (Dutta and Sengupta, 2016). In mice, the firsts signs of puberty are reported at the 4 weeks of age (~postnatal day [PD] 28) (Dutta and

Sengupta, 2016); however, the average age at which mice reach puberty is about PD 42 (1.5 months) and the mean puberty age in the human being is about 11.5 years (Dutta and Sengupta, 2016).

4.1.2. Adulthood

The age at which mice or other animals attain sexual maturity is defined as adulthood (Dutta and Sengupta, 2016). However, in humans there are other psychological and social concepts that are associated with this stage.

The sexual maturity in mice is reached at 8-12 weeks of age, with an average of 10 weeks (~PD 70). In humans, a physical change that allows to differentiate between the adolescence and adulthood is the close of the growth plates in the scapula, which is around 20 years of age (Dutta and Sengupta, 2016).

Reproductive senescence is characterized by the termination of the fertility cycle. In mice, although the biomarkers of ageing are not yet detected, the cease of reproductive functions begin in middle age (10-14 months) which correlates with the human age of 38-47 years of age (The jackson laboratory, 2017). In humans, the mean age of women reproductive senescence is around 51 years old (Dutta and Sengupta, 2016).

4.1.3. Senescence

The period in which biomarkers of aging can be detected in almost all animals is the senescence or old age. In mice, senescence is determined by at least 18 months until the end of the lifespan (around 24 months)

(Dutta and Sengupta, 2016; The jackson laboratory, 2017). In human, the old age start more or less at 56 years old (The jackson laboratory, 2017).

4.2. Maternal Separation as a model of early-life adversity

As previously exposed, the effects of several factors in the human child development could be studied in mice from the day of birth up to post-natal day (PD) 28. There are several animal models to explore early-life stress and/or early-life adversity, however, most of them have in common the alteration in the amount and/or quality of maternal care, due to is the principal factor that could has impact in the early postnatal period of humans and also other animals (Krugers et al., 2017).

The most common animal paradigm to study the early-life adversity is the maternal separation (Baracz et al., 2020; George et al., 2010; Vetulani, 2013). This model consist on separating the pups from the dam repeatedly over the first postnatal days, allowing to evaluates the consequences of early-life adversity during the postnatal or neonatal period (Schmidt, 2010).

Maternal separation is a reliable animal model of early life neglect (George et al., 2010), but also a good model of major depression disorder (Planchez et al., 2019; Vetulani, 2013).

There are several studies that employs different periods of maternal separation but the most popular experimental procedure of maternal separation consists of a 3 h daily separation from the second to the PD12 (Planchez et al., 2019).

George et al (2010) performed several studies evaluating if 3 h of maternal separation from PD 2 to PD 17, had long-lasting effects on behaviour. The results showed no significant effect of maternal separation on several behavioural tests. Therefore, they tried with 3 h of separation from PD 2 to PD 17 and weaned at PD 17. Early weaning showed the reduction of compensatory maternal care after maternal separation; however, the results continue to be inconsistent. For these reasons, they developed a new model joining longer periods of maternal separation and early weaning, in order to reduce the maternal contact but ensuring the safety and viability of the offspring (George et al., 2010). The final model was named the maternal separation with early weaning (MSEW), and includes periods of 4 h of maternal separation, from PD 2 to PD 5 and 8 h of separation from PD 6 to 16. Offspring was subsequently weaned at PD 17, instead PD 21. The combination of these variables ensures a good reproducibility and repeatability of the results.

At a behavioural level, this kind of experimental manipulation induces behavioural abnormalities such as learning and memory performance deficits, depressive-like behaviours and anxiety-like behaviours, higher reactivity to stress during adulthood, anhedonia and a proclivity for increased drug consumption in future develop stages (Planchez et al., 2019; Tractenberg et al., 2016).

5. Maternal Separation with Early Weaning

5.1. Behavioural outcomes

5.1.1. Maternal care

As previously argued, maternal care is critical during the postnatal period. This behaviour makes reference to the behaviour performed by the dam to nourish and protect her litter during its early development (Orso et al., 2019). There are a variety of measures to allow evaluates the maternal care, such as: licking/grooming, arched-back nursing, blanket-nursing/passive nursing, nest building, harmful/adverse caregiving (off-nest behaviour) (Orso et al., 2019).

Evaluation of the arched-back nursing, blanket posture and off-nest behaviour in MSEW mice reveals that MSEW mothers exhibited the arched-back nursing and the blanket posture more often than the standard nest (SN) mothers (Gracia-Rubio et al., 2016b). In the case of the off-nest measure, MSEW mothers spent less time out of the nest than SN mothers (Gracia-Rubio et al., 2016b). Moreover, no changes on body weight, male/female ratio, mortality, morbidity or metabolic derangements, were observed in MSEW pups (George et al., 2010; Gracia-Rubio et al., 2016b).

As a consequence of the disruption of the maternal care, studies report increased basal levels of corticotropin-releasing factor (CRF), higher levels of corticosterone, ACTH, and CRF, inducing a hyperactivity of the HPA axis. These alterations underlie the development of stress-induced mental health disorders such as anxiety-like and depression-like behaviours (Baracz et al., 2020).

5.1.2. Locomotor activity

In other hand, locomotor assays are used to determine the total motor activity of an animal over a given time period (Carter and Shieh, 2015).

The level of net activity can be altered by depression in human patients or also in animal models (Planchez et al., 2019).

Measured of spontaneous locomotor activity showed that MSEW mice of both sexes, performed decreased deambulations and rearings during adolescence period (Gracia-Rubio et al., 2016b), however, no significant differences were observed during adulthood (Gracia-Rubio, 2016).

Analysis of open field behaviour, a classical test to provide a qualitative and quantitative measurement of exploratory and locomotor activity in rodents (Valvassori et al., 2017), revels that MSEW mice moves faster and covers more area than the controls (George et al., 2010). This result demonstrate that MSEW animals showed hyperactivity compared with the control mice (Valvassori et al., 2017).

5.1.3. Anxiety-like behaviour

Moreover, the open field test is also used to test anxiety-like behaviour due to the animal is exposed to an unknown environment whose escape is prevented by surrounding walls (Gogas et al., 2007). Studies also showed that MSEW mice spent more time in the centre of the open field than the controls (George et al., 2010). This result display a higher novelty-seeking and risk-taking behaviour, due to rodents typically prefer not to be in the centre and tend to walk close to the walls (Gogas et al., 2007; Valvassori et al., 2017).

Another behavioural test to measure anxiety is the elevated plus maze (EPM). This test uses nonpainful, non-aversive stimuli to induce fear and anxiety, reducing the motivational and perceptual states (Gogas et al., 2007). The apparatus consist on four elevated arms (two open and

two close) in the shape of a "plus" signal (Gogas et al., 2007). Normal animal behaviour is avoiding the open arms, spending relatively more time in the closed arms (Carter and Shieh, 2015). Evidence show that MSEW mice have decrease time and entries in open arms than SN animals during adolescence and also during adulthood (Gracia-Rubio et al., 2016b). In line with these, George et al (2010) also reported that higher number of open arm entries was significantly lower for MSEW than control animals, but no significant effect of MSEW on average time spent in a closed arm. Together, these results showed that MSEW mice showed an increased anxiety-like behaviour.

5.1.4. Despair-like behaviour

Common test for assessing despair-like behaviour include forced swim test and tail suspension test (TST) (Planchez et al., 2019). These behaviour test evaluates a hopelessness state in rodents, placing them in an uncomfortable situation from which is impossible to scape (Planchez et al., 2019; Valvassori et al., 2017). At the beginning of both test, there is a period of active behaviour (swimming or struggling) during the animal tries to scape (Carter and Shieh, 2015; Planchez et al., 2019). However, after this period of motor agitation, animals exhibit immobility, indicating the animal had learned that escape is impossible (Valvassori et al., 2017). This immobility is taken as an indication of behavioural despair and is commonly considered to reflect depression-like states (Planchez et al., 2019; Valvassori et al., 2017). From an anthropomorphically view, this behaviour is interpreted as loss of hope in a stressful situation (Valvassori et al., 2017).

Gracia-Rubio et al. (2016) reported that MSEW adolescent and adult CD1 mice spent increased immobile time in the TST compared with the SN group. Portero-Tresserra et al (2018) also observed increased immobility time in adolescent C57BL/6 mice that experienced MSEW. In the forced swim test, results showed that MSEW mice spent significantly more time immobile than the control group (George et al., 2010).

In sum, these studies exhibited that MSEW induced long lasting behavioural changes that increased depressive-like symptoms in mice, being a good animal model of depression (Ménard et al., 2016; Vetulani, 2013).

5.1.5. Anhedonia

Anhedonia is the inability or diminished capacity to feel pleasure from normally rewarding stimuli and is a core symptom of depression (Cryan and Slattery, 2010).

The most common model to measure anhedonia in rodents is the sucrose/saccharin preference test (Carter and Shieh, 2015). In general, rodents have the choice between water solution or sucrose/saccharin solution (Carter and Shieh, 2015; Planchez et al., 2019). Normally, animals show preference for the saccharin solution, which is significantly reduced in models of depression (Carter and Shieh, 2015; Planchez et al., 2019).

Saccharin preference test showed that MSEW mice have decreased preference for saccharine compared with the SN animals during the adolescence period, however, during adulthood they did not observe

significative difference between the groups (Gracia-Rubio et al., 2016b). In line with this, Frank et al. (2019) also reported significantly lower sucrose preference in maternally separated rodents and higher submissive behaviour in resident intruder and dominant-submissive tests.

On other hand, Matthews and Robbins (2003) showed that maternal separation alter the response to heroin and raclopride, a selective antagonist of D2R, showing that this kind of early-life stress induce a predisposition to anhedonia.

5.1.6. Addiction

We said that MSEW shower higher novelty-seeking and risk-taking behaviour and this behaviour has been associated with predisposition to rewarding and addictive behaviours (Valvassori et al., 2017). Moreover, as exposed in point 5.1.4, MSEW is also an animal model of depression-like behaviour, a factor that can trigger or intensify the desire to drug use (Koob and Le Moal, 2008, 2001; Rappeneau and Bérod, 2017).

Animal models have shown that early-life stress exposure increases the consumption of several drugs, including cocaine (Bagley et al., 2019b).

Several studies report that maternal separation enhances the acquisition of cocaine SA (Lynch et al., 2005; Moffett et al., 2006), higher cocaine-induced hyperlocomotion (Kikusui et al., 2005), increases cocaine intake and elevates the number of responses during the reinstatement phase (Lynch et al., 2005).

Lynch, Mangini and Taylor, (2005) showed that early-life stress potentiates cocaine seeking in male- and female- rats (Lynch et al., 2005); however, during the extinction phase of cocaine SA, stressed males showed a higher levels of responding than stressed females (Lynch et al., 2005).

Mice (both sexes) exposed to maternal separation evidenced greater cocaine-induced locomotor stimulation than mice no maternally separated (Kikusui et al., 2005). However, only maternally separated males, no females, developed behavioural sensitization to cocaine challenges (Kikusui et al., 2005). An important observation is that females were more sensitive to the effects of acute cocaine injections, expressed higher levels of GR and dopamine transporter (DAT) in the NAc, independently of the maternal separation procedure (Kikusui et al., 2005).

In contrast, Gracia-Rubio et al. (2016) reported that mice exposed to maternal separation diminished cocaine-induce sensitization and no changes in the rewarding effects of cocaine (CPP) in adolescent mice, while Viola et al. (2016) stated that maternal separation potentiates cocaine-induced CPP in animals at PD 45, together with decreased gene expression of BDNF.

Gracia-Rubio et al. (2016) also observed that maternally separated mice expressed a reduction in D2R in the NAc and elevated expression of the transcriptional factor Nurr1 in the VTA (Gracia-Rubio et al., 2016a). These alterations were accompanied by increased dopamine turnover and protein expression of DAT in the NAc of maternally separated mice exposed to cocaine (Gracia-Rubio et al., 2016a). In line with this

observation, Romano-López et al. (2015) showed that maternal separation facilitates alcohol ingestion due to deregulation of the dopaminergic system in the PFC and the NAc, increasing the dendritic length, the expression of D2R and the tyrosine TH levels.

Finally, cocaine-induced CPP decreased the gene expression of miR-212 and elevated the expression of Mecp2 gene level in the PFC of animals reared normally, while MSEW CPP-experienced, did not showed alteration neither mir-212 nor Mecp2 (Viola et al., 2016). Therefore, maternal separation disrupts compensatory mechanisms in the PFC associated with cocaine rewarding effects, leading to increased cocaine-induced CPP (Viola et al., 2016).

Taken together, these evidence display that maternal separation increases behavioural and neurochemical responsiveness to cocaine and interacts with sex to impact the psychostimulant and rewarding effects of cocaine (Bagley et al., 2019b, 2019a). Therefore, MSEW is a reliable animal model to understand why early-life adversity increases the vulnerability to substance use disorders.

5.1.7. Glutamatergic synaptic plasticity

Maternal separation can induce long-lasting effects in several brain pathways. Hsu et al. (2003) observed that this kind of early-life stress modifies the hippocampal GABA receptors function and subunit expression during adulthood. These maternally separated rats maintained an immature GABAergic phenotype accompanied by increased activity in response to swim stress (Hsu et al., 2003).

Pickering et al. (2006) determined changes induced by maternal separation in the hippocampus and PFC of rats. They observed lower mRNA expression of NMDA NR2B, AMPA GluA1 and GluA2 subunits in the hippocampus but any changes in the PFC. Two hypotheses were proposed to explain these alterations. The first one is that the expression of these genes changed as a homeostatic mechanism in order to adapt to the chronic stress exposition; the second one is that, the overactivation of glutamate receptors in the hippocampus due to chronic stress, the neurons died by the increased excitotoxicity (Pickering et al., 2006).

In contrast, other study reported impairment in the LTP and increased protein expression of GluA1, GluA2, postsynaptic density protein 95 and CAMKII in the mPFC of maternally separated rats (Chocyk et al., 2013). These results correlated with higher anxiety in the light/dark exploration test and decrease in spine density in apical and basal dendrites of pyramidal neurons of the mPFC (Chocyk et al., 2013).

Ganguly et al. (2019) evaluated the effects of maternal separation on cocaine-induced CPP in adolescent male- and female rats. They observed a male-specific effect of maternal separation, due to only males formed a preference for a cocaine-paired environment in a subumbral dose (10 mg/kg). This behavioural change was in line with a loose of tumour necrosis factor (TNF)-mediated GluA2-containing AMPA receptors expression in the PFC and NAc (Ganguly et al., 2019). In line with this, another study also observed sexual dimorphism in the expression of GluA1 and GluA2 mRNA levels in the hippocampus and amygdala of rats (Katsouli et al., 2014).

In addition to the modifications induced to the AMPA receptor composition, cocaine exposure and MSEW also alters molecular substrates of plasticity (Bowers et al., 2010). Among the molecular changes reported that emerge in abstinence period, we can mention the CREB and the BDNF (Bowers et al., 2010).

Dixon et al. (2019) reported changes in BDNF expression in the hippocampus, VTA and PFC of maternally separated rats (Dixon et al., 2019). This could explained the higher vulnerability to addiction, due to increased BDNF in the VTA of rats turns the GABA inhibitory system to excitatory signalisation, increasing the probability of drug abuse (Enoch, 2011).

Lippmann et al. (2007) showed an increased BDNF level in the VTA as well as a decreased expression in the striatum and hippocampus of maternally separated rats. An interesting observation is that reduce BDNF in the hippocampus (Duman and Monteggia, 2006) and elevated BDNF in the VTA (Eisch et al., 2003), induce a depression-like phenotype in rats. Bian et al. (2015) also reported increases immobility time in the forced swim test and TST, together with reduced mRNA and protein levels of BDNF in the hippocampus.

Related with CREB, rodents show that CREB overexpression in the NAc decreases rewarding effects of cocaine (Carlezon et al., 1998). A study evaluating long-term effects of maternal separation on the depression related behaviour reported a decrease in BDNF and CREB protein levels in the hippocampus (Shu et al., 2015). These molecular modifications were in hand with decreases in sucrose preference and spontaneous locomotor activity (Shu et al., 2015).

However, there are other authors that failed to find CREB modifications in the NAc induced by maternal separation (Sachs et al., 2018), VTA (Lippmann et al., 2007), NAc (Lippmann et al., 2007) or hippocampus (Lippmann et al., 2007). Nevertheless, it must exist alteration in this transcription factor, due to CREB regulates BDNF transcription in a phosphorylation-dependent manner in the brain (Lippmann et al., 2007).

A possibility is that the studies cited, measured CREB but no the changes in the phosphorylation of this important transcription factor, which are more relevant to its role as a transcription activator (Lippmann et al., 2007). Confirming this observation, Bian et al. (2015) reported that maternal separation did not modify the protein level of CREB but induced a significative decline in pCREB, which is the active form.

5.1.8. Sex differences in behaviour

A recent review about the impact of early-life stress in rodent studies (Orso et al., 2019) showed that only 46.2% of rat studies and 29.4% of mice studies performed sex difference comparison. Moreover, only 38.4% of the studies in rats and 64.7% in mice, used only male animals (Orso et al., 2019).

Tractenberg et al. (2016) also evaluates the characteristics of several mice studies in which the maternal separation protocol was used. They reported that 52.08% included both sexes, 41.66% employed only males, 2.08% used only females, and 4.16% did not report the sex of the animals. Besides, in this study they sum the behavioural and

physiological findings by sex and strain, however, for a practical purpose, we grouped the results of all the strains in one graph **(Figure 14)**.

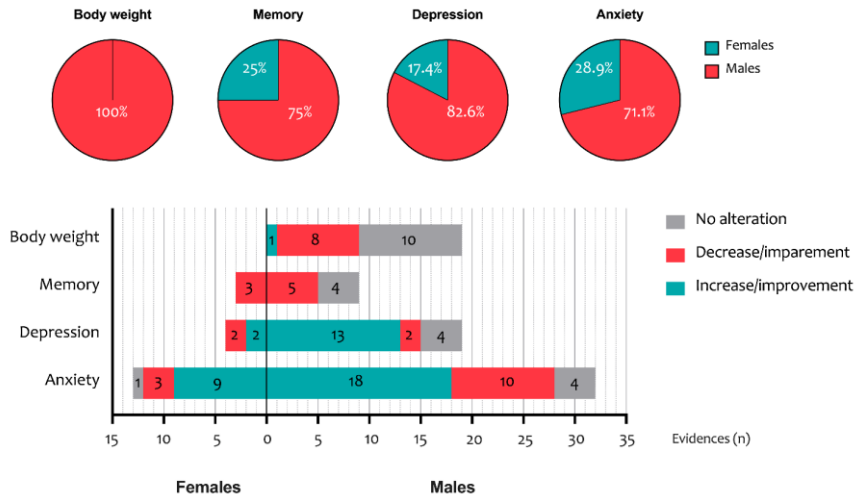


Figure 14. Sex differences for behavioural and physiological findings from separation models in mice. Adapted from: Tractenberg et al. (2016).

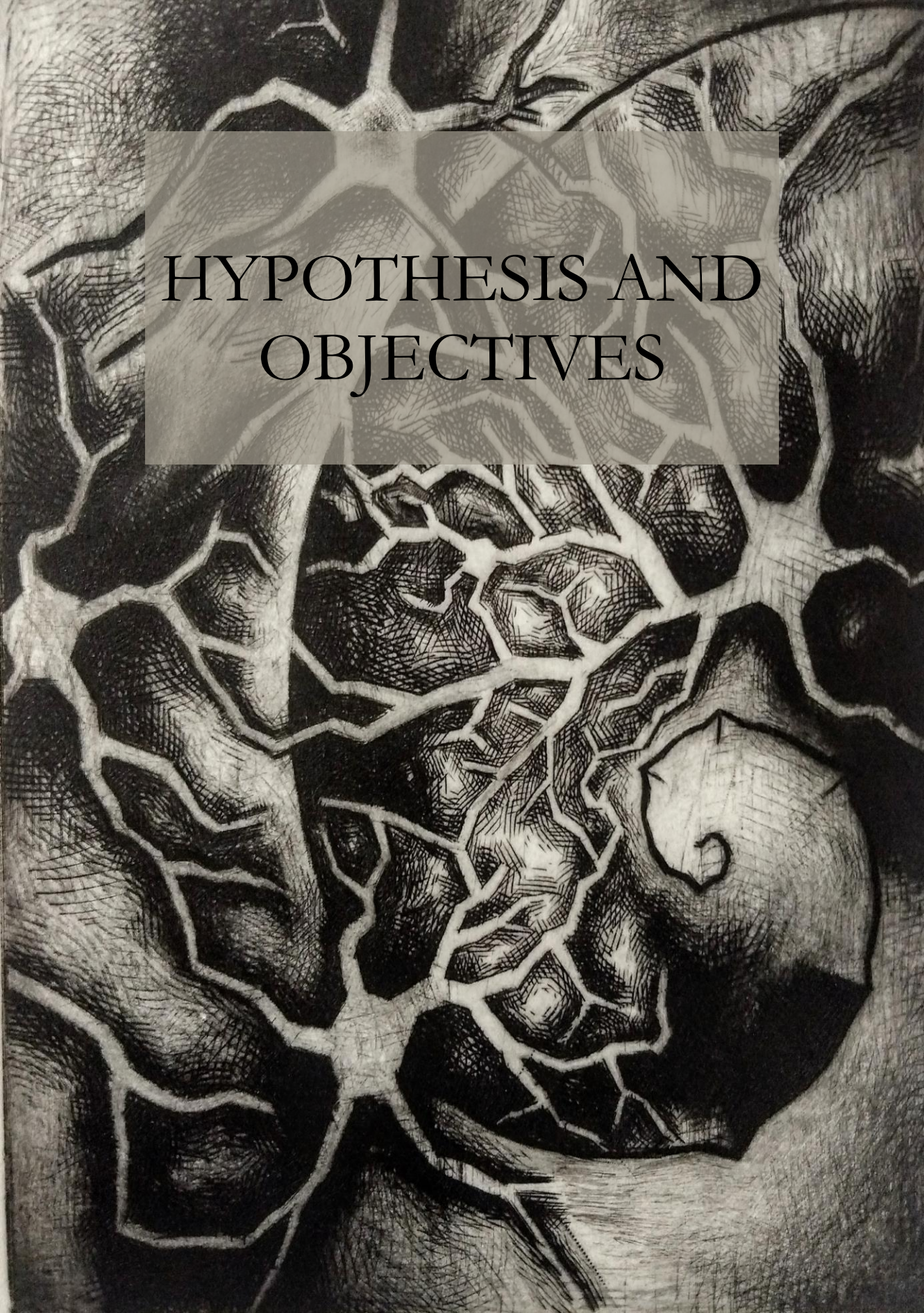
Analysis of the data show that 71.1% of the studies in maternally separated mice evaluating anxiety were performed in males, and only 28.9% of the measures were done in female mice. Here, the results seem to be homogenous, due to the great percentage of the measures showing increased anxiety-like behaviour in males (56.3%) and female mice (69.2%).

The sex-specific evidence for depression-like behaviour was derived predominantly from studies with male mice (82.6%) rather than female mice (17.4%). In this case, the results are heterogeneous because maternal separation trend to increase the depression-like behaviour in

males (68.4%) while in females the 50% reported increased- and 50% decreased depression-like behaviour.

Once again, in studies evaluating memory performance, most of the data come from males (75%) and a small percentage from females (25%). However, in both cases, the trend is that maternal separation induces memory impairments.

Orso et al. (2019) remarks the importance to use both sexes, because there is a lot of evidence that demonstrated that dams tend to spend more time actively nourishing males than female pups. Therefore, the alterations in maternal care could affect in a different way the stress response of the pups depending on the sex (Orso et al., 2019).



HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Considering the theoretical frame that we have exposed in the introduction, we hypothesized that early-life stress, induced by MSEW, evocates several molecular alterations in the AMPA receptor subunits composition and other molecules related to neuroplasticity which persist until adulthood. Because of these alterations, animals exposed to early-life stress will show higher vulnerability to impulse control disorders including CUD.

OBJECTIVES

The overall objective of this thesis was to assess in mice, the influence of MSEW on cocaine abuse during adulthood, and the alterations on GluA2, GluA1, CREB and pCREB, induced by early-life stress and/or cocaine exposure. To this end, we used experimental approaches to explore this possibility, including behavioural studies and biochemical analysis.

In order to achieve our main aim, the following specific objectives are proposed:

Objective 1

To study in male mice, the long-term effects of MSEW on the molecular substrate related to neuroplasticity and depressive-like behaviour, as well as the contribution of such molecular alterations during the acquisition and reinstatement of cocaine SA behaviour.

a) To describe behavioural consequences of MSEW in the reinforcing effects of cocaine using the operant SA

paradigm during acquisition, extinction and reinstatement phases.

b) To evaluate changes induced by MSEW on the basal levels of GluA1, GluA2, CREB and pCREB, in the VTA and NAc.

c) To determine changes on GluA1, GluA2, CREB and pCREB in the VTA and the NAc, after the acquisition and reinstatement phases of cocaine SA paradigm.

Objective 2

To investigate sex differences in the effects of MSEW during the acquisition of cocaine SA behaviour and the molecular changes induced by cocaine exposure and/or early-life stress.

a) To study differences between males and females previously exposed to MSEW in the acquisition of cocaine SA behaviour.

b) To evaluate if MSEW induces sex-specific changes on the basal levels of GluA1, GluA2, CREB and pCREB in the VTA and NAc.

c) To explore changes on the levels of GluA1, GluA2, CREB and pCREB in the VTA and NAc after the acquisition of cocaine SA, as a possible explanation for the telescoping effect.

Objective 3

To study the MSEW consequences on emotional alterations and the underlying molecular changes in the mPFC of male and female mice.

a) To study the MSEW effects on despair-like behaviour using the TST.

b) To analyse the MSEW effects on Gria1 and Gria2 mRNA levels in the mPFC.

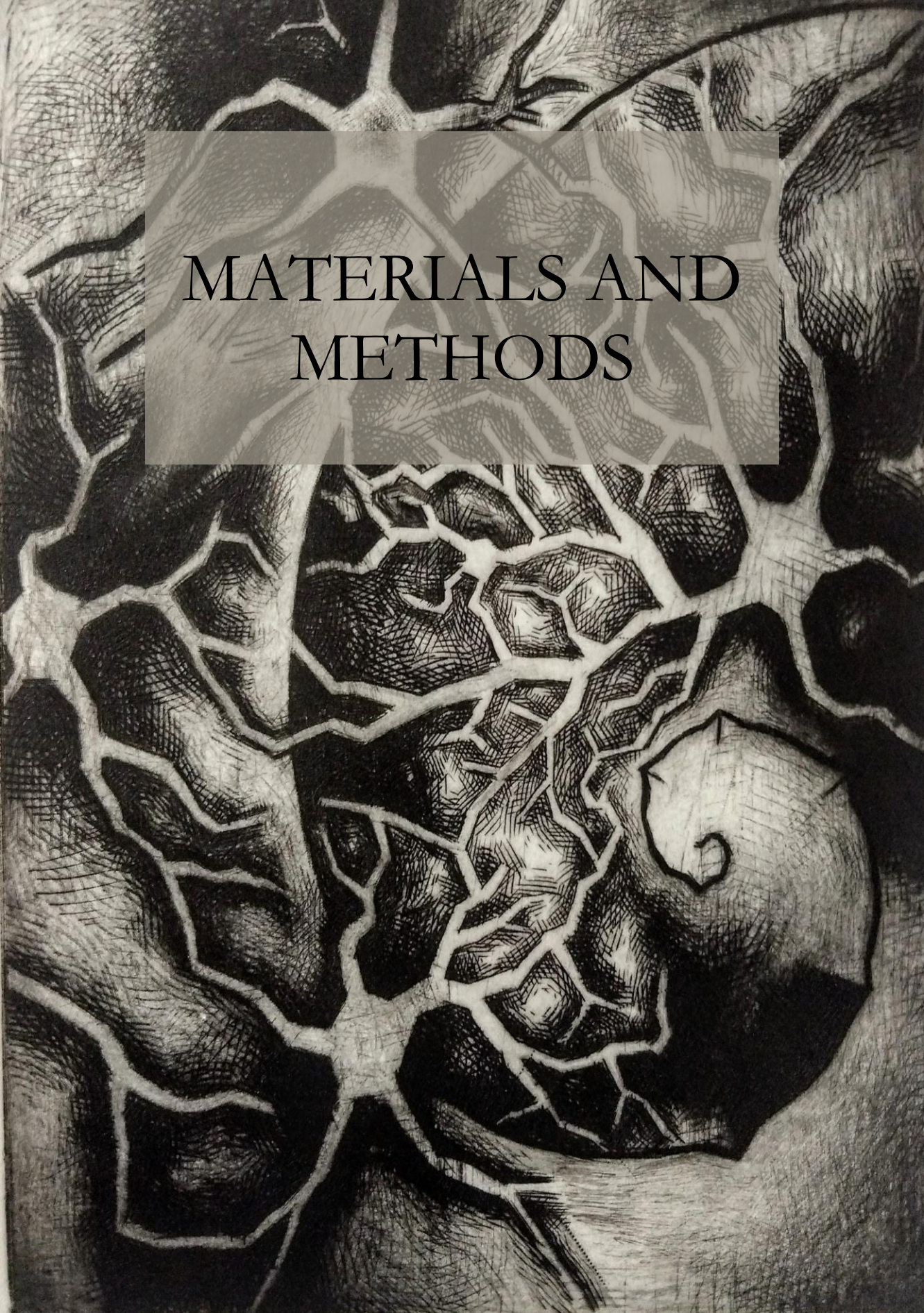
c) To determine a correlation between despair-like behaviour and Gria2 expression.

Objective 4

To explore sex-variations in the impulsivity of MSEW mice in order to explain the difference in the acquisition of cocaine SA, as well as the molecular alterations of AMPA receptor subunit composition in the mPFC.

a) To assess changes in the impulsivity for food and cocaine seeking using the operant SA.

b) To determine alterations of GluA1 and GluA2 in the mPFC of cocaine self-administering mice.



MATERIALS AND METHODS

1. Animals

CD1 adult mice aged 10 weeks used as breeders (Charles River, Barcelona, Spain), were received at the animal facility, UBIOMEX, PRBB. The animals were placed in pairs in standard cages in a temperature- (21 ± 1ºC) and humidity- (55% ± 10%) controlled room and subjected to a 12 h light/dark cycle with the lights on from 8:00 to 20:00 h, with *ad libitum* access to food and water. Ten days later, the males were removed, and we maintained the same conditions for the pregnant females. We carried the experiments out under the guidelines of the European Communities Directive 88/609/EEC regulating animal research. All procedures were approved by the local ethical committee (CEEA-PRBB) and all efforts were made to minimise the animal suffering and to decrease the number of animals used.

2. Rearing conditions

The rearing conditions were as previously described (Castro-Zavala et al., 2020; Gracia-Rubio et al., 2016b; Portero-Tresserra et al., 2018). New-born mice were randomly assigned to the experimental groups: SN or MSEW. The day of birth was considered the PD 0. Animals in the MSEW were separated from their mothers for 4 h per day (9:00 to 13:00 h) from PD 2 to PD 5, and 8 h per day (9:00 to 17:00 h) from PD 6 to PD 16. For the separation, the mother was removed and placed in another cage and room, leaving the pups in their home box. To maintain the body temperature of the pups, home boxes were placed upon electric blankets until the mother was duly returned. Animals in the SN remained with their mother until weaning (PD 21), whilst animals in the MSEW were weaned at PD 17. In both cases (SN and MSEW), cages were cleaned on PD 10. We distributed the pups of each litter between

the different experimental groups in order to avoid a litter effect. MSEW procedure does not affect body weight (Gracia-Rubio et al., 2016b; Portero-Tresserra et al., 2018), mortality (George et al., 2010), morbidity (George et al., 2010), or the male/female ratio (Koob and Zorrilla, 2010)

3. Drugs

Cocaine was purchased from Alcatel (Ministry of Health, Madrid, Spain) and was dissolved in sterile physiological saline (0.9%). A 1 mg/kg infusion dosage of cocaine was used for the acquisition phase of the SA procedure and 10 mg/kg (i.p) to induce reinstatement.

4. Behavioural tests

4.1. Tail suspension test

Mice underwent the TST on PD 60 as previously described (Gracia-Rubio et al., 2016b). Briefly, each mouse was suspended 50 cm above a benchtop for 6 minutes (using adhesive tape attached 1cm from the tip of the tail). The time (s) that the animal was immobile during this interval was recorded.

4.2. Cocaine self-administration

Surgery. Surgeries were performed on PD 53-57. The surgery for the intravenous catheter implantation was performed following anaesthetization with a mixture of ketamine/xylazine (50 mg/mL, 10 mg/mL, administrated in a volume of 0.15 mL/10g), animals were implanted with the jugular catheter. We treated animals with analgesic (Meloxicam 0.5 mg/kg; i.p., administrated in a volume of 0.10 mL/10g)

and antibiotic solution (Enrofloxacin 7.5 mg/kg; i.p., administrated in a volume of 0.03 mL/10 g). After surgery, animals were housed individually, placed over electric blankets, and allowed to recover.

Acquisition. At least 3 days after surgery, we trained animals on a fixed ratio 1 to self-administer cocaine (1.0 mg/kg per infusion) during 10-day sessions (2 h each). The number of nosepokes (responses during time in and time out) and the number of infusions (responses during time in), in the active- and the inactive hole, were counted. We considered mice to have acquired stable SA behaviour when the following criteria were met on 2 consecutive days: ≥5 responses on the active hole and ≥ 65% of responses on the active hole.

Extinction. Only the animals meeting acquisition criteria continued to the extinction phase. The experimental conditions were the same as in the acquisition phase with the exception that the active hole had no consequences, and the stimulus light was always turned off. The extinction criteria were: ≤30% of active responses than the day of maximum consumption during the acquisition phase, on two consecutive days. The patency of the i.v. catheters was evaluated at the end of the first extinction session by an infusion of 0.1 mL of tiobarbital (thiopental sodium; 5 mg/mL; i.v.; B. Braun Medical, S.A. Rubí, Barcelona, Spain). If we observed no signs of anaesthesia within the first 3s, we excluded the mouse from the experiment.

Reinstatement. We induced reinstatement by a cocaine priming injection (10 mg/kg; i.p) only in the animals meeting extinction criteria (24 h after reaching criteria). Immediately after being injected, we placed the mice in the SA box to begin the reinstatement test. The reinstatement session

was identical to the acquisition sessions, although the animals did not receive the drug infusions.

4.3. Food self-administration

Four days before testing commenced (PD56), SN and MSEW mice were food-restricted, being fed accordingly to the 95% of their body mass daily. Food restriction lasted the duration of food-maintained operant behaviour. Water was available *ad libitum* during the experimental phase. The animals were trained, on a fixed ratio 1, to nosepoke for food pellets (Grain-Based Rodent #5001, Test Diet, Sawbridgeworth, UK) for 10-day sessions (2 h each). The nosepoke number in the active- and the inactive hole (responses during time in and time out) and the pellet number (responses during time in) were counted.

4.4. Percentage of response efficiency and motor impulsivity

The evaluation of motor impulsivity could be divided into two processes: impulsive action and impulsive choice (Dalley et al., 2011; Dalley and Ersche, 2019). Impulsive action is measured by the incapacity to self-restraint and perform anticipatory actions, known as a failure of motor inhibition (Dalley et al., 2011; Dalley and Ersche, 2019).

Adapting the formula employed by Hynes et al. (2018) to our SA paradigm, we calculated the percentage of response efficiency as an indirect measure of impulsivity. A 100% response efficiency represents

enough responses to obtain the reinforcement (one food pellet/one nosepoke or one cocaine infusion/one nosepoke). Therefore, decreased response efficiency means an increased impulsive response.

For the cocaine SA, we used the following formula:

$$\%\ Response\ efficiency = \frac{Number\ of\ cocaine\ infusions}{Number\ of\ active\ nosepokes} \times 100$$

In the case of food SA, we calculated the percentage of response efficiency as follows:

$$\%\ Response\ efficiency = \frac{Number\ of\ food\ pellets}{Number\ of\ active\ nosepokes} \times 100$$

As a result, mice with high percentage response efficiency scores were considered less impulsive, while mice with low percentage response efficiency were considered more impulsive (Hynes et al., 2018).

5. Biochemical assays

5.1. Animal Sacrifice and Sample Collection

Animals were sacrificed by cervical dislocation. Brains were immediately removed from the skull and placed in a cold plaque. Samples were dissected at different times. For Article 1, VTA and NAc samples from SN and MSEW mice, were obtained in basal conditions (drug-naïve), after acquisition (the last day of the acquisition phase) and on reinstatement day (30 minutes after the reinstatement session). For

Article 2, we dissect the NAc and the VTA from drug-naïve and drug-experienced mice of both sexes (30 minutes after the final acquisition day). For Article 3, we obtained the mPFC in drug-naïve condition, after cocaine SA (drug-experienced) and after TST (PD60).

Samples taken after acquisition were exclusively from the mice that acquired the cocaine SA behaviour. In all the cases, the samples were immediately stored at -80ºC until the biochemical analysis was performed.

5.2. Western Blot

To evaluate the expression of GluA2, GluA1, CREB and pCREB, 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). Protein samples (16 μg) were mixed with 5X loading buffer (TRIS pH 6.8 0.153 M, SDS 7.5%, Glycerol 40%, EDTA 5 mM, 2-β-mercaptoethanol 0.025%, bromophenol blue 0.025%), loaded and run on SDS-PAGE 10% and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with bovine serum albumin (BSA) 5% for 1 h at room temperature and incubated overnight at 4ºC with primary antibodies **(Table 2)**. To detect primary antibodies, we used fluorescent secondary antibodies **(Table 2)** incubated for 1 h at room temperature. Images were acquired on a Licor Odyssey Scanner and quantified using Image Studio Lite software v5.2 (LICOR, USA).

Table 2. Antibodies used in western blot

Antibody	# Catalogue	RRIDs	Dilution	Company
GluA2	AB1768	AB_2313802	1:1000	Milipore
GluA1	ABN241	AB_2721164	1:1000	Milipore
β-tubulin	556321	AB_396360	1:5000	BD Biosciences
goat anti-mouse IgG H&L (IRDye 800)	ab216772	AB_2857338	1:2500	Abcam
goat anti-rabbit IgG H&L (DyLight 680)	611-144-002	AB_1660962	1:2500	Rockland

For Article 1, the expression of GluA2, GluA1, CREB, pCREB and β-tubulin, was evaluated in the VTA and NAc of males. The different groups were: SN-basal, MSEW-basal, SN-acquisition, MSEW-acquisition, SN-reinstatement and MSEW-reinstatement (n=5, run in triplicate). Data were normalised to control group (SN) to ascertain the changes due to MSEW, and cocaine exposure at different phases or the combination of both factors.

For Article 2, the expression of GluA2, GluA1, CREB, pCREB and β-tubulin was evaluated in the VTA and NAc of males and female mice. The groups were: SN drug-naïve males, SN drug-experienced males, MSEW drug-naïve males, MSEW drug-experienced males, SN drug-naïve females, SN drug-experienced females, MSEW drug-naïve

females and MSEW drug-experienced females (n=5 per group, run in duplicate or triplicate). Firstly, we analysed the levels of GluA2, GluA1, CREB and pCREB (in drug-naïve animals), only normalizing to β-tubulin (optical density, OD). Subsequently, data were normalized to the control group (SN drug-naïve) of each sex in order to ascertain the fold change due to MSEW, cocaine exposure or the combination of both variables.

For Article 3, the expression of GluA1, GluA2 and β-tubulin were evaluated in the mPFC of males and females. The groups were: SN drug-naïve males, SN drug-experienced males, MSEW drug-naïve males, MSEW drug-experienced males, SN drug-naïve females, SN drug-experienced females, MSEW drug-naïve females and MSEW drug-experienced females (n=4-5 per group, run in triplicate). Data were normalized to the SN naïve males in order to determine the fold change due to sex, MSEW, cocaine exposure or the interaction between variables.

5.3. RNA isolation and real-time PCR

For this procedure, we used mPFC samples of SN and MSEW mice (males and females), at basal condition or after the TST. We extract the RNA using trizol as previously described (Cardenas-Perez et al., 2018). RT-PCR was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using random primers and following standardized protocols.

5.4. Quantitative PCR for Gria1 and Gria2

For the qPCR, we use cDNA of mPFC samples (20 ng), Light Cycler SBYR green 480 Master Mix (Roche LifeScience, Product No. 04707516001) and the specific primers **(Table 3)** for Gria1, Gria2 and 36B4 as housekeeping gene (Integrated DNA Techologies, Inc.). The qPCR was performed in LightCycler ® 480 Instrument II (Roche LifeScience) using next program: 95°C-10s, 60°C-20s, 72°C-10s for 45 cycles.

Table 3. Primers for qPCR

	Primer sequence (5'→3')
Gria1 Forward	ACA ACT CAA GCG TCC AGA ATA G
Gria1 Reverse	CAT AGC GGT CAT TGC CTT CA
Gria2 Forward	CCT TTC TTG ATC CTT TAG CCT ATG A
Gria 2 Reverse	CTG CTG ACC AGG AAT AAA ACT ACA CT
36B4 Forward	TCC AGG CTT TGG GCA TCA
36B4 Reverse	CTT TAT CAG CTG CAC ATC ACT CAG A

6. Statistical analysis

In all the cases, we analysed data for conditions of normality (Kolmogorov-Smirnov`s test), sphericity (Mauchly`s test) and homoscedasticity (Levene`s test).

For Article 1, we analysed data regarding the infusions during the acquisition phase using a two-way repeated measures ANOVA (*rearing* and *days*). In the case of nosepokes during the acquisition of cocaine SA, a three-way repeated measures ANOVA was applied (*rearing, hole* and *days*). For the extinction phase, a two-way repeated measures ANOVA was calculated for each group (SN and MSEW) based on the following factors: *rearing* and *hole*. In the case of reinstatement, we analysed the nosepokes registered on the last extinction day compared with the nosepokes on reinstatement day. For this comparison, a three-way repeated measures ANOVA was calculated with *rearing, hole* and *days* (last extinction day vs reinstatement day) as factors of variation. In the case of percentages, Fisher`s exact test was used. The results of total cocaine intake, day of acquisition and day of extinction, were calculated using an unpaired two-tailed Student´s t-test. We analysed western blot results through a two-way ANOVA with *rearing* and *phase* (control, acquisition and reinstatement) as independent factors, and the expression of target proteins as a dependent variable.

For article 2, data for infusions were analysed by means of a four-way repeated measures ANOVA based on the following factors: *days*, *hole* (active or inactive), s*ex* and r*earing*. Data for acquisition day, total cocaine intake and acquisition percentage were analysed using two-way ANOVA with s*ex* and *rearing* as inter-subject variables. For western blot results, the OD of drug-naïve animals was analysed using a student´s t test using *rearing* as a variable. Subsequently, the fold change of each molecule was analysed using a three-way ANOVA with *rearing, sex* and *phase* as independent factors. When F achieved $p<0.05$, the ANOVA was followed by the Bonferroni post-hoc test if a main effect and/or

interaction was observed. All possible post-hoc comparisons were evaluated. All statistical analyses were performed using SPSS Statistics v23. Data were expressed as mean ± SEM and a value of $p<0.05$ was considered significant.

For Article 3, data from the TST, qPCR, average of response efficiency and western blot results of drug-naïve mice were analysed using a two-way ANOVA with *rearing* and *sex* as independent factors. Western blot results of drug-naïve and drug-experienced animals were analysed using a three-way ANOVA with *rearing*, *sex* and *treatment* as factors. Data of percentage of response efficiency were analysed using a three-way ANOVA repeated measures with *rearing*, *sex* and *days* as factors of variation.

In all the cases, the ANOVA was followed by the Bonferroni post-hoc tests only if F achieved $p<0.05$. 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.

RESULTS

Article 1

Maternal separation increases cocaine intake through a mechanism involving plasticity in glutamate signalling.

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Addict Biol. 2020 Apr 24:e12911.

DOI: https://doi.org/10.1111/adb.12911

Received: 6 May 2019 | Revised: 5 April 2020 | Accepted: 8 April 2020
DOI: 10.1111/adb.12911

ORIGINAL ARTICLE

Addiction Biology SSA/RED WILEY

Maternal separation increases cocaine intake through a mechanism involving plasticity in glutamate signalling

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Funding information
Ministerio de Sanidad, Grant/Award Numbers: Plan Nacional sobre Drogas 2018/007, Retic-ISCIII, RD16/017/010, RD16/017/010, Retic-ISCIII; Consejo Nacional de Ciencia y Tecnología, Gobierno de Mexico, Grant/Award Number: 276577; Ministerio de Economía y Competitividad, Grant/Award Numbers: FPU 15/02492, SAF2016-75966-R-FEDER

Abstract

Early-life stress (ELS) is associated with negative consequences, including maladaptive long-lasting brain effects. These alterations seem to increase the likelihood of developing substance use disorders. However, the molecular consequences of ELS are poorly understood. In the present study, we tested the impact of ELS induced by maternal separation with early weaning (MSEW) in CD1 male mice at different phases of cocaine self-administration (SA). We also investigated the subsequent alterations on GluR2, GluR1, cAMP response element-binding (CREB), and CREB-phosphorylation (pCREB) in ventral tegmental area (VTA) and nucleus accumbens (NAc) induced by both MSEW and cocaine SA. Our results show that MSEW animals expressed a higher cocaine intake, an increased vulnerability to the acquisition of cocaine SA, and incapacity to extinguish cocaine SA behaviour. MSEW mice showed decreased GluR2 and increased GluR1 and pCREB in NAc. Also, results displayed reduction of basal levels of GluR1 and CREB and an elevation of GluR1/GluR2 ratio in the VTA. Such results hint at an enhanced glutamatergic function in NAc and increased excitability of VTA DA neurons in maternally separated mice. Altogether, our results suggest that MSEW induces molecular alterations in the brain areas related to reward processing, increasing the vulnerability to depression and cocaine-seeking behaviour.

KEYWORDS
cocaine self-administration, CREB, GluR1, GluR2, maternal separation with early weaning, pCREB

1 | INTRODUCTION

Early-life stress (ELS) during prenatal and postnatal periods is associated with negative consequences, including maladaptive long-lasting brain effects. The estimation is that one in four children is victim of abuse or negligence, and this could increase the risk for developing psychiatric disorders. Epidemiologic studies report that adults with a history of childhood adversity show 28.4% increased risk of depression and 16.5% to illicit drug use. Animal models have shown that ELS exposure increases the consumption of several drugs, including cocaine. In rodents, maternal separation (MS) with early weaning (MSEW) is a reliable animal model of ELS and depression-like behaviour. Other studies have reported changes in brain-derived neurotrophic factor (BDNF) expression in the hippocampus, ventral tegmental area (VTA), and prefrontal cortex of maternally separated rats. Moreover, increased BDNF in the VTA of rats induces a higher vulnerability to drug abuse, turning the GABA inhibitory system to excitatory signalization.

Among the illicit drugs, cocaine is one of the most consumed with over 18 million cocaine users, and approximately 20% of them will

shift from cocaine use to cocaine use disorder (CUD). Several studies have indicated that cocaine induces aberrant neuroplastic changes in the mesolimbic system, affecting different neurotransmitter systems including the glutamatergic function, and such alterations may be related to CUD and cocaine relapse. Among the cocaine-induced glutamatergic changes, the alteration in AMPA receptor (AMPAr) subunit composition has been recently studied. AMPAr is made up of four subunit proteins (GluR1-GluR4) and generally composed of GluR2 in complex with GluR1 or GluR3. However, GluR2-lacking AMPAr is calcium-permeable, inducing higher synaptic strengthening. Previous data have shown an increased GluR2 subunit expression in the nucleus accumbens (NAc) and VTA of cocaine overdose victims. Moreover, increased levels of GluR2 and GluR1 in the NAc of cocaine self-administering rhesus monkeys have also been reported. Studies in rats have disclosed increased GluR1 levels in the VTA following cocaine self-administration (SA) and enhanced motivation for cocaine-seeking behaviour. However, previous studies have shown no changes in GluR1 or Glur2 levels in the VTA nor the NAc after cocaine SA.

One of the major challenges for CUD clinical treatment is the high relapse rate following abstinence (<45%). Animal models of relapse have shown that the cocaine-primed reinstatement of cocaine SA enhances the activation of glutamatergic projection from the prefrontal cortex to the NAc, facilitating cocaine-seeking behaviour and the reinforcing effects of cocaine. Furthermore, an increase of GluR2-lacking AMPAr in the NAc after withdrawal intensifies cocaine relapse and cocaine-seeking.

There is a significant comorbidity between CUD and other neuropsychiatric disorders, in particular major depression. Recently, we demonstrated that MSEW enhanced susceptibility to cocaine addiction and cocaine intake in males but not in female mice. Other studies in maternally separated rodents reported an enhanced vulnerability to cocaine SA, as well as higher cocaine-induced hyperlocomotion. GluR2-lacking AMPAr has been suggested as a regulator of depression-like behaviour. Likewise, modifications in GluR2-lacking AMPA transmission in the VTA and NAc have been reported as a common target linking addiction and mood disorders. In maternally separated rats, alterations in the GluR2 and GluR1 gene and protein expression were observed in the hippocampus and the prefrontal cortex. Besides to GluR2 and GluR1 alterations, cocaine exposure and MSEW also modified molecular substrates of plasticity. Studies in rodents show that cAMP response element-binding (CREB) overexpression in the NAc decreases rewarding effects of cocaine. Indeed, repeated cocaine administration decreases CREB and increases CREB-phosphorylation (pCREB) in the NAc, attenuating the rewarding effects of the drug. Moreover, CREB overexpression in the VTA increases sensitivity to cocaine rewarding effects. Additionally, increased pCREB expression in the VTA of mice was observed after cocaine priming, inducing reinstatement to CPP in accordance with the up-regulation of CREB observed in the VTA of cocaine overdose victims. In relation to MSEW, previous studies in rodents using the combination of MS and social defeat models have found that the exposure to social defeat modulated CREB activity and BDNF levels but failed to find CREB modifications in the NAc due to MS.

In view of the impact that ELS has in the developing of cocaine abuse, it is necessary to explore the neurobiological mechanisms to find a possible clinical target to avoid the deleterious effects of this kind of stress.

Until now, the levels of GluR2, GluR1, CREB, and pCREB in vulnerable individuals to CUD are still unknown. The study of changes in molecular substrates of plasticity could help to explore the comorbidity between depression and CUD. Moreover, the changes in these molecules at different stages of the addiction process in vulnerable individuals could shed light on new therapeutic strategies. Hence, we investigated in mice the long-term effects of MSEW in factors related to neuroplasticity, depression, and cocaine exposure. The aim of our study was to assess the influence of MSEW on GluR2, GluR1, CREB, and pCREB in VTA and NAc. Furthermore, we evaluated the contribution of such molecular alterations and MSEW during the acquisition and reinstatement of cocaine SA behaviour.

2 | MATERIALS AND METHODS

2.1 | Animals

Fourteen male and 14 female CD1 adult mice aged 10 weeks used as breeders (Charles River, Barcelona, Spain) were received at the animal facility, UBIOMEX, PRBB. The animals were placed in pairs in standard cages in a temperature- (21 ± 1°C) and humidity- (55% ± 10%) controlled room and subjected to a 12-hour light/dark cycle with the lights on from 8:00 to 20:00 hours, with ad libitum access to food and water. Ten days later, the males were removed, and we maintained the same conditions for the pregnant females. We carried the experiments out under the guidelines of the European Communities Directive 88/609/EEC regulating animal research. All procedures were approved by the local ethical committee (CEEA-PRBB), and all efforts were made to minimize the animal suffering and to decrease the number of animals used.

2.2 | Rearing conditions

The rearing conditions were as previously described (Figure 1A). Newborn mice were randomly assigned to the experimental groups: standard nest (SN) and MSEW (Figure 1A). The day of birth was considered the postnatal day (PD) 0. We have distributed the pups of each litter between the different experimental groups to avoid a litter effect. We separated animals in the MSEW group from their mothers for 4 hours per day (9:00 to 13:00 h) from PD2 to PD5 and 8 hours per day (9:00 to 17:00 h) from PD6 to PD16 (see Supporting Information).

2.3 | Drugs

Cocaine was purchased from Alcatel (Ministry of Health, Madrid, Spain) and was dissolved in sterile physiological saline (0.9%). A

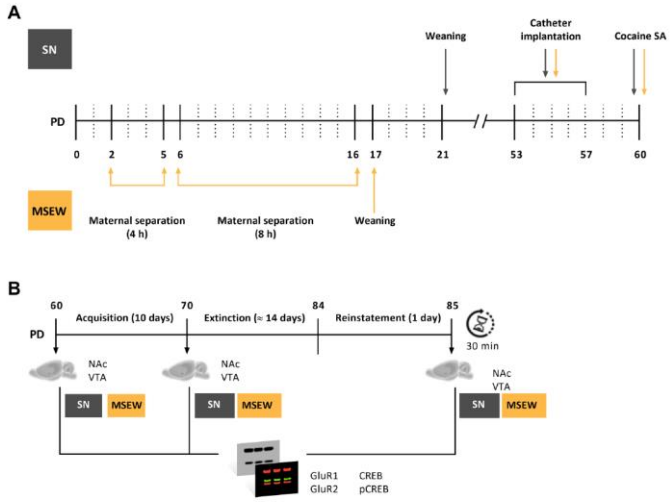


FIGURE 1 Schematic representation of the experimental schedule. (A) Schematic representation of the maternal separation with early weaning (MSEW) model and the (B) timeline in which the brain samples were obtained

1-mg/kg infusion dosage of cocaine was used for the acquisition phase of the SA procedure and 10 mg/kg (i.p) to induce reinstatement.

2.4 | Operant cocaine SA

2.4.1 | Surgery

We conducted the SA experiments as previously described in Castro-Zavala et al. Briefly, when animals (SN = 30 mice, MSEW = 46 mice) reached the PD 53 to 57, a jugular-vein catheter implantation was performed (see Supporting Information). Following surgery, animals were housed individually, placed upon electric blankets, and allowed to recover.

2.4.2 | Acquisition

At least 3 days after surgery, we trained animals on a fixed ratio 1 to self-administer cocaine (1.0 mg/kg per infusion) during 10 daily sessions (2 h). See Supporting Information for details. The number of nosepokes (responses during time in and time out) and the number of infusions (responses during time in), in the active and the inactive holes, were counted. We considered mice to have acquired stable SA behaviour when the following criteria were met on two consecutive days: ≥5 responses on the active hole and ≥65% of responses on the active hole.

2.4.3 | Extinction

Only the animals meeting acquisition criteria continued to the extinction phase. See Supporting Information for details. The number of extinction sessions was variable depending on each animal (maximum 14 sessions). The extinction criteria were ≤30% of active responses than the day of maximum consumption during the acquisition phase, on two consecutive days. The patency of the i.v. catheters was evaluated at the end of the first extinction session by an infusion of 0.1 mL of tiobarbital (thiopental sodium; 5 mg/mL; i.v.; B. Braun Medical, S.A. Rubí, Barcelona, Spain). If we observed no signs of anaesthesia within the first 3 seconds, we excluded the mouse from the experiment.

2.4.4 | Reinstatement

We induced reinstatement by a cocaine priming injection (10 mg/kg; i.p) only in the animals meeting extinction criteria (24 h after reaching

criteria). Immediately after being injected, we placed the mice in the SA box to begin the reinstatement test. See Supporting Information for details.

2.5 | Animal euthanasia and sample collection

Animals were euthanized by cervical dislocation, and the brains were immediately removed from the skulls and placed in a cold plaque. Brain samples from SN and MSEW mice were dissected at different periods: basal conditions (nontreated animals), after acquisition (the last day of the acquisition phase), and on reinstatement day (30 min after the reinstatement session) (Figure 1B). Cerebellum and olfactory bulbs were discarded, and the VTA and NAc were dissected (see Supporting Information). The areas were immediately stored at −80°C for posterior western blot assays.

2.6 | Western blot for GluR2, GluR1, CREB, and pCREB

To test the expression of GluR2, GluR1, CREB, and pCREB, samples were homogenized in cold lysis buffer (Table S1), supplemented with protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). Protein samples (16 μg) were mixed with 5X loading buffer (Table S2), loaded and run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis 10% and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with bovine serum albumin 5% for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies (Table S3). Primary antibodies were detected with fluorescent secondary antibodies (Table S4), incubated for 1 hour at room temperature. Images were acquired on a Licor Odyssey Scanner and quantified using Image Studio Lite software v5.2 (LICOR, USA). The expression of GluR2, GluR1, CREB, pCREB, and β-tubulin was evaluated in the VTA and NAc of the different groups: SN-basal, MSEW-basal, SN-acquisition, MSEW-acquisition, SN-reinstatement, and MSEW-reinstatement (n = 5, run in triplicate). Data were normalized to control group (SN) to ascertain the changes due to MSEW, and cocaine exposure at different phases or the combination of both factors.

2.7 | Statistical analysis

We analysed data for conditions of normality (Kolmogorov-Smirnov's test), sphericity (Mauchly's test), and homoscedasticity (Levene's test). We analysed data regarding the infusions during the acquisition phase using a two-way repeated measures analysis of variance (ANOVA) (***rearing*** and ***days***). In the case of nosepokes during the acquisition of cocaine SA, a three-way repeated measures ANOVA was applied (***rearing***, ***hole***, and ***days***). For the extinction phase, a two-way repeated measures ANOVA was calculated for each group (SN and MSEW) on the basis of the following factors: ***rearing*** and ***hole***. In the case of reinstatement, we analysed the nosepokes registered on the last extinction day compared with the nosepokes on reinstatement day. For this comparison, a three-way repeated measures ANOVA was calculated with ***rearing***, ***hole***, and ***days*** (last extinction day vs. reinstatement day) as factors of variation. In the case of percentages, Fisher's exact test was used. The results of total cocaine intake, day of acquisition, and day of extinction were calculated using an unpaired two-tailed Student's t test. We analysed western blot results through a two-way ANOVA with ***rearing*** and ***phase*** (control, acquisition, and reinstatement) as independent factors and the expression of target proteins as a dependent variable. Bonferroni post-hoc tests were run only if F achieved $P < .05$. All statistical analyses were performed using SPSS Statistics v23. Data were expressed as mean ± standard error of the mean, and a value of $P < .05$ was considered statistically significant.

3 | RESULTS

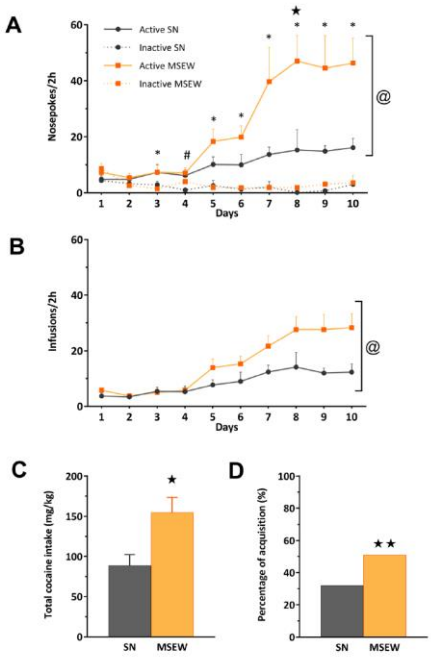
3.1 | MSEW increases cocaine SA during the acquisition phase

Only the animals who met acquisition and extinction criteria were registered for infusion and nosepoke analysis (SN = 7, MSEW = 13). Three-way repeated measures ANOVA for the nosepokes in the acquisition phase (Figure 2A) showed a main effect of ***rearing*** ($F_{1,18}$ = 9.476, $P < .01$), ***days*** ($F_{9,162}$ = 5.310, $P < .001$), and ***hole*** ($F_{1,18}$ = 40.226, $P < .01$); an interaction between ***days*** and ***hole*** ($F_{9,162}$ = 6.194, $P < .001$) and ***hole*** and ***rearing*** ($F_{1,18}$ = 7.996, $P < .05$); and an interaction between the three factors ($F_{9,162}$ = 2.078, $P < .05$). Bonferroni ***post-hoc*** for the interaction between all factors showed that the MSEW group discriminated between the active and inactive hole on day 3 and, in a stable manner, from day 5 to day 10, with significantly higher responses on the active hole ($P < .05$ in all the cases). Conversely, the SN group only discriminated between the active and inactive holes on day 4 ($P < .05$). The post-hoc test also revealed that the MSEW group made significantly more active nosepokes on day 8 when compared with the SN group ($P < .05$).

When we analysed the number of cocaine infusions, two-way repeated measures ANOVA (Figure 2B) showed an effect of ***rearing*** ($F_{1,18}$ = 5.750, $P < .05$) and ***days*** ($F_{9,162}$ = 10.377, $P < .001$). The ***rearing*** effect revealed that the MSEW group obtained a higher number of infusions than the SN group.

Student's t-test analysis for total cocaine intake during the acquisition phase revealed higher cocaine consumption in the MSEW than in the SN group (t_{18} = 2.398, $P < .05$) (Figure 2C). We found no significant difference for the day of acquisition (data not shown).

Considering the criteria of acquisition, the percentage of mice acquiring the cocaine SA behaviour was 32% (n = 7/22) for SN and 51% (n = 20/41) for MSEW (Figure 2D). Fisher's exact test shows a significantly higher percentage of acquisition in the MSEW group than in the SN group ($P < .01$).



F IGURE 2 Effects of maternal separation with early weaning (MSEW) on the acquisition of cocaine self-administration behaviour. Mean of nosepokes (A) and infusions (B) in the active/inactive holes during the 10-day acquisition phase in the standard nest (SN) (n=7) and (MSEW) (n=13) group. (C) Mean of total cocaine intake (mg cocaine mg/kg mice) through the acquisition phase (SN n=7; MSEW n=13). (D) Percentage of animals reaching acquisition criteria (SN n=7/22; MSEW n=20/41). Discrimination between active/inactive holes in MSEW (*P < 0.05) and SN (#P < 0.05) group; rearing effect (★P < 0.05, ★★P < 0.01, @P < 0.001). Bonferroni post-hoc test. Data are expressed as mean ± standard error of the mean.

3.2 | Maternally separated mice display a lower extinction rate in the cocaine SA paradigm

We used only the animals meeting the acquisition and extinction criteria for the infusion and nosepoke analyses (SN = 7, MSEW = 13). As expected, active nosepokes declined during the extinction sessions in both groups (Figure 3). Two-way ANOVA for the SN group (Figure 3A) reveals an effect of *days* ($F_{12,\ 106}$ = 4.117, P < .001), *hole* ($F_{1,106}$ = 22.334, P < .001), and interaction between the two factors ($F_{12,106}$ = 2.574, P < .01). Bonferroni post-hoc test shows a significant discrimination between the active and inactive holes for the first 4 days (P < .05 in all the cases). In the case of the MSEW group (Figure 3B), two-way ANOVA shows an effect of *days* ($F_{12,\ 106}$ = 4.251, P < .001), *hole* ($F_{1,106}$ = 37.564, P < .001), and interaction between both factors ($F_{12,106}$ = 2.803, P < .01). Bonferroni post-hoc analysis reveals significant discrimination between the active and the inactive hole only for the first 5 days of the extinction phase (P < .05 in all the cases). In accordance with the extinction criteria, Fisher's exact test reveals that all the SN mice (n = 7/7) extinguished cocaine SA behaviour, while only 65% of the MSEW mice (n = 13/20) met the extinction criteria (P < .001) (Figure 4B). We found no significant difference for the day of extinction (data not shown). We also observed that MSEW mice tended to respond more in the extinction phase (P = .057) when compared with SN mice (162% more than the SN group in terms of total responses) (data not shown).

3.3 | Cocaine priming-induced reinstatement of drug seeking in both SN and MSEW mouse groups

The day after the animals extinguished, we administered an i.p. cocaine priming to evaluate the reinstatement of drug-seeking behaviour. A three-way ANOVA revealed a main effect of *rearing* ($F_{1,18}$ = 6.863, P < .05), *days* (last extinction day and reinstatement day) ($F_{1,18}$ = 17.405, P < .01), *hole* ($F_{1,18}$ = 12.150, P < .01),

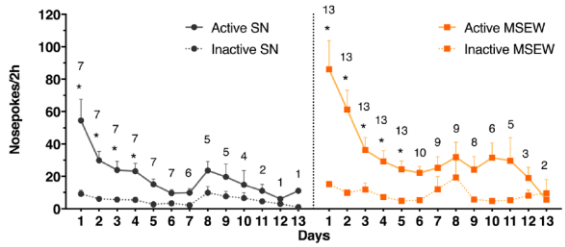


FIGURE 3 Extinction of cocaine self-administration behaviour. Mean of active/inactive nosepokes during the extinction phase in standard nest (SN) (A) and maternal separation with early weaning (MSEW) (B) group. Discrimination between active and inactive (*P < .05). The number above each day represents the number of animals using for the analysis. Animals missing are the ones that completed the extinction criteria. Data are expressed as mean ± standard error of the mean (SN n = 7; MSEW n = 13)

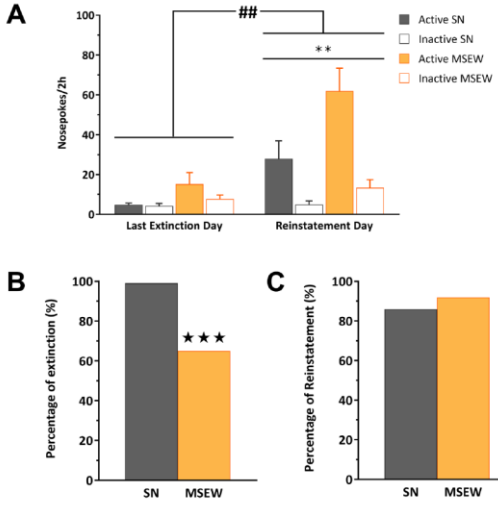


FIG URE 4 Effects of maternal separation with early weaning (MSEW) on cocaine-induced reinstatement, percentage of extinction and percentage of reinstatement. (A) Mean of responses in the active/inactive holes on the last extinction day and reinstatement day (SN n=7; MSEW n=13). (B) Percentage of mice reaching the extinction criteria (SN n=7/7; MSEW n=13/20). (C) Percentage of mice reinstating cocaine self-administration behaviour (SN n=6/7; MSEW n=12/13). Days effect (##P < 0.01); discrimination between active/inactive holes (**P < 0.01); rearing effect (★★★P < 0.001). Bonferroni post-hoc test. Data are expressed as mean ± standard error of the mean.

and interaction between ***days*** and ***hole*** ($F_{1,18}$ = 14.739, P < .01) (Figure 4A). The ***rearing*** effect demonstrated that MSEW animals presented a greater number of responses in comparison with the SN mice, showing higher cocaine-seeking in this group of mice (P < .05). Bonferroni post-hoc analysis for the interaction showed that cocaine priming increased the active nosepokes during the reinstatement day in comparison with the final extinction day (P < .01). Also, between-hole discrimination was clear on reinstatement day (P < .01). As shown by Fisher's test, the percentage of cocaine reinstatement in the SN group (86%, n = 6/7) did not differ significantly from the MSEW group (92%, n = 12/13) (Figure 4C).

3.4 | MSEW decreased GluR2 and increased both GluR1 and the GluR1/GluR2 ratio in the NAc

Two-way ANOVA for GluR2, GluR1, and GluR1/GluR2 (Figure 5A) showed a main effect of *rearing* ($F_{1,24}$ = 7.022, P < .05), ($F_{1,23}$ = 4.530, P < .05), and ($F_{1,23}$ = 12.640, P < .01), respectively. Such results indicate that MSEW decreased the expression of GluR2 and increased both GluR1 and the GluR1/GluR2 ratio. We got no significant *phase* effect or interaction between factors either for GluR2, GluR1, or GluR1/GluR2.

3.5 | Increased CREB-phosphorylation induced by MSEW and decreased CREB expression in SN group after acquisition of cocaine SA behaviour in the NAc

Two-way ANOVA for CREB expression shows the interaction between *rearing* and *phase* ($F_{1,24}$ = 3.369, P < .05). The post-hoc test revealed a significantly decreased CREB expression after the acquisition phase compared with the basal levels in the SN group (P < .01) (Figure 5B). The main effects failed to reach significance. In the case of pCREB (Figure 5B), the results only revealed a main effect of *rearing*

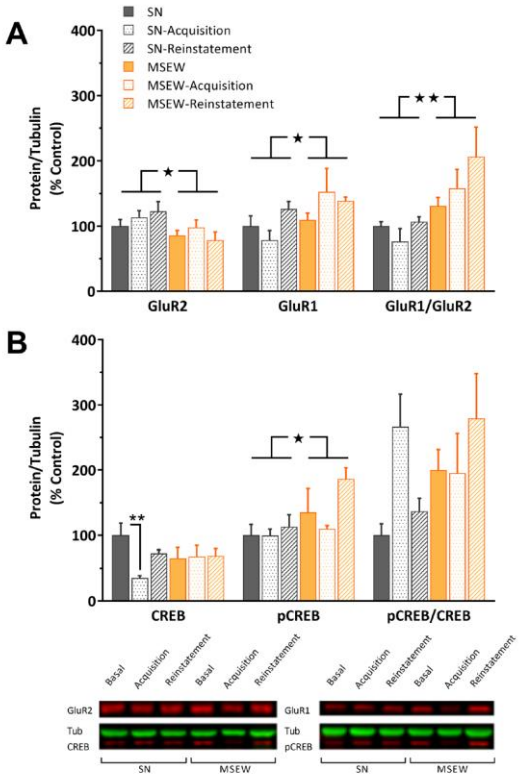


FIGURE 5 Western blot analyses in the nucleus accumbens (NAc) of standard nest (SN) and maternal separation with early weaning (MSEW) mice at different phases of the cocaine self-administration behaviour. (A) Mean of GluR2, GluR1 and GluR2/GluR1 levels. (B) Mean of CREB, pCREB and pCREB/CREB levels. Rearing effect (★P < 0.05, ★★P < 0.01) and Bonferroni post-hoc comparison indicated with the lines (**P < 0.01). Data are expressed as mean ± standard error of the mean (n=5, run in triplicate).

($F_{1,24}$ = 6.058, P < .05), showing that MSEW had an increased pCREB expression compared with SN. No significant changes were observed in the pCREB/CREB ratio.

3.6 | Cocaine primed-reinstatement increased the expression of GluR2 and decreased the GluR1/GluR2 ratio in the VTA of MSEW mice

Two-way ANOVA for GluR2 (Figure 6A) demonstrated a main effect of *phase* ($F_{1,23}$ = 7.914, P < .01) and the interaction between *phase* and *rearing* ($F_{1,23}$ = 5.429, P < .05). The *phase* effect shows an increased expression of GluR2 during reinstatement compared with the basal levels (P < .01). The Bonferroni post-hoc test showed a significant basal reduction of GluR2 in the MSEW animals compared with the basal level in the SN mice (P < .05) and a significant increase of GluR2 after reinstatement in the MSEW in comparison with the basal levels (P < .001) and also when compared with the levels in the acquisition phase respectively (P < .05). For GluR1 (Figure 6A), two-way ANOVA showed a *phase* effect ($F_{1,23}$ = 10.375, P < .01), with a decreased expression of GluR1 after acquisition, when compared with the basal levels (P < .01). Also, a significant increase was observed

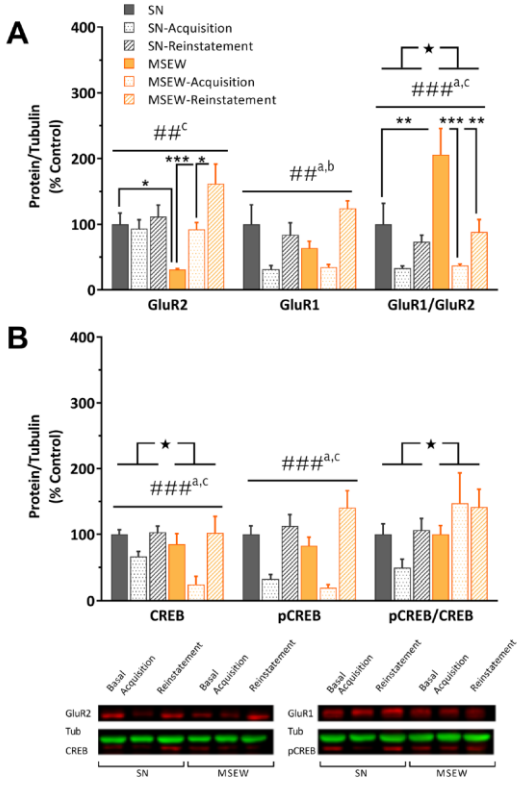


FIG URE 6 Western blot analyses in the ventral tegmental area (VTA) of standard nest (SN) and maternal separation with early weaning (MSEW) mice at different phases of the cocaine self-administration behaviour. (A) Mean of GluR2, GluR1 and GluR2/GluR1 levels. (B) Mean of CREB, pCREB and pCREB/CREB levels. Rearing effect (★P < 0.05), phase effect (##P < 0.01, ###P < 0.001; a=basal vs. acquisition, b=acquisition vs. reinstatement, c=basal vs. reinstatement) and Bonferroni post-hoc comparison indicated with the lines (*P < 0.05, **P < 0.01, ***P < 0.001). Data are expressed as mean ± standard error of the mean (n=5, run in triplicate).

after reinstatement in comparison with levels in the acquisition phase ($P < .01$). The ***rearing*** effect or interaction between factors did not reach significance. In the case of the GluR1/GluR2 ratio (Figure 6A), the two-way ANOVA revealed a main effect of ***rearing*** ($F_{1,23} = 5.845$, $P < .05$), ***phase*** ($F_{1,23} = 15.400$, $P < .001$), and the interaction between factors ($F_{1,23} = 3.361$, $P < .05$). The ***rearing*** effect shows that MSEW mice have a higher GluR1/GluR2 ratio compared with the SN group. The ***phase*** effect reveals a significant decrease in the GluR1/GluR2 ratio during acquisition and reinstatement, when compared with the basal levels ($P < .01$). The Bonferroni post-hoc test shows the MSEW group to have a higher GluR1/GluR2 ratio under basal conditions in comparison to the SN group ($P < .01$). In the MSEW group of mice, the GluR1/GluR2 ratio decreased after acquisition ($P < .001$) and reinstatement when compared with the basal ratio ($P < .01$).

3.7 | Changes in CREB and pCREB expression in VTA after the acquisition phase of the cocaine SA behaviour

Two-way ANOVA for CREB (Figure 6B) showed ***rearing*** ($F_{1,23} = 7.639$, $P < .05$) and ***phase*** ($F_{1,23} = 10.960$, $P < .001$) effects. The ***rearing*** effect showed a decreased CREB expression in MSEW compared with SN mice ($P < .05$), while the ***phase*** effect demonstrated a decrease in CREB expression after acquisition ($P < .01$) and a subsequent CREB increase after reinstatement when compared with the acquisition phase ($P < .01$). For pCREB, two-way ANOVA revealed a significant ***phase*** effect ($F_{1,23} = 17.416$, $P < .001$). This main effect showed a significant decrease in pCREB following acquisition ($P < .01$) and increased pCREB expression after reinstatement ($P < .001$), compared with basal levels. The two-way ANOVA for the pCREB/CREB ratio (Figure 6B) revealed a main effect of ***rearing*** ($F_{1,23} = 5.200$, $P < .05$). This effect showed MSEW mice to have a higher pCREB/CREB ratio in comparison with the SN group ($P < .05$). ***Phase*** and interaction between ***phase*** and ***rearing*** failed to reach statistical significance.

4 | DISCUSSION

Our findings show that MSEW increases the reinforcing effects of cocaine in the SA paradigm, including the percentage of the acquisition, cocaine intake, number of nosepokes, and infusions performed during the acquisition phase. Moreover, MSEW mice extinguished cocaine-seeking behaviour in a lower percentage and no changes were appreciated in terms of reinstatement. The analysis of protein expression (GluR1, GluR2, CREB, and pCREB) involved in plasticity during the addictive process revealed significant changes in the expression of such molecules following the acquisition and reinstatement of cocaine SA in the NAc and VTA of MSEW mice. As far as we know, this is the first study investigating molecular changes associated with MSEW and cocaine-induced neuroplasticity in male mice exposed to cocaine SA.

Previously, Romano-López et al (2015) showed that MS induced several changes in the prefrontal cortex and in the NAc, suggesting a deregulation of the dopaminergic system that facilitates alcohol ingestion. Regarding the NAc, Romano-López et al (2015) showed that MS increases dopamine D2 receptor (D2R) expression, tyrosine hydroxylase (TH) levels and the dendritic length. In agreement with these results, we previously displayed alterations in the mesolimbic dopamine system in our model of MSEW. We reported a decreased expression of D2R in the NAc and higher expression of Nurr1 in the VTA, a transcription factor that activates the dopamine synthesis and the transcription of dopamine transporter protein, vesicular monoamine transporter, and TH. Additionally, we observed that following cocaine-induced CPP, the levels of Nurr1 in the VTA decreased significatively, in accordance with clinical studies that report decreased expression of Nurr1 in midbrain dopaminergic neurons of cocaine abusers. Accordingly to previous studies, MS upregulated the DNA methyltransferases in the NAc of adult rats, inducing an hypermethylation of genes taking part in cocaine-induced neuroplasticity mechanisms. Moreover, Larsen et al (2016) reported that Nurr1 gene shows a CpG islands with higher GC content in the promoter region, meaning that DNA methylation regulates the transcription of Nurr1, concurring with our previous results. Therefore, our results support that changes induced by the MS in the mesolimbic dopamine system contribute to generate detrimental neuroadaptations in the brain.

In accordance with our findings, previous studies have shown that MS enhances the acquisition of cocaine SA, increases cocaine intake, and elevates the number of responses during the reinstatement phase, thus supporting the view that ELS increases vulnerability to cocaine addiction. In humans, approximately 20% of cocaine users become addicts, but the likelihood of shifting from cocaine use to CUD increases two folds in individuals with a history of childhood maltreatment. Indeed, while the percentage of SN mice acquiring cocaine SA was 32%, the rate increased to 51% in MSEW mice. Additionally, epidemiologic studies reveal that childhood maltreatment negatively impacts cocaine relapse. Thus, whereas the cocaine relapse rate is approximately 45% for individuals who have suffered childhood maltreatment, the figure rises to around 90%. Although our results show a similar reinstatement percentage for both groups of mice, cocaine-seeking behaviour during reinstatement was higher in the MSEW group.

Previously, we reported that MSEW induced anhedonia, higher anxiety- and depressive-like behaviour in mice. In line with such data, Goffer et al (2013) reported an increased GluR1 expression in the NAc of depressive rats, pointing to GluR2-lacking AMPAr as a regulator of depressive-like behaviours. Moreover, a higher depression vulnerability was associated with an increased GluR1/GluR2 ratio (increased AMPAr function) and decreased GluR2 levels in the NAc in socially-stressed mice. Accordingly, our results reveal a decreased GluR2 expression and an increased GluR1 expression in the NAc of MSEW mice. Furthermore, studies in rats showed an increased GluR1 levels and a higher GluR1/GluR2 ratio in the NAc after repeated cocaine exposure. Besides, GluR2-lacking AMPA receptors are

associated with an augmented excitability of NAc neurons, increased cocaine craving, and a higher reinstatement of previously extinguished cocaine-seeking behaviour. Likewise, increased GluR1 in the NAc of rats is associated with higher reinstatement, and consistently, our results show that MSEW mice exhibited a higher GluR1 expression and higher cocaine-seeking behaviour during all the phases of cocaine SA.

We therefore propose that the NAc-glutamatergic function of MSEW mice is facilitated due to decreased GluR2 expression, increased GluR1 expression, and a higher GluR1/GluR2 ratio. Such facts could potentiate the inhibitory function of GABAergic neurotransmission from the NAc to the VTA, thus inhibiting the dopamine release from VTA neurons. This decreased dopamine levels will affect to other brain structures than the NAc, such as frontal cortex, hippocampus, amygdala, and also the VTA per se. As a result of this enhanced inhibition, MSEW mice may increase their cocaine intake to achieve greater drug-reinforcement effects, showing higher susceptibility to cocaine craving, cocaine seeking, and cocaine reinstatement. However, we cannot dismiss that independent mechanisms could cause glutamatergic alterations in both structures.

In accordance with our hypothesis, previous studies showed that cyanquixaline (CNQX), an AMPAr antagonist, administrated locally in discrete brain areas or systemically, are able to reduce cocaine intake and cocaine-seeking behaviour. For example, infusions of CNQX into the core of the NAc decreased levels pressing for cocaine in rats and blocked the reinstatement of cocaine seeking induced by intra–prefrontal cortex cocaine infusion in rats. Additionally, Mahler et al (2013) showed that microinjections of CNQX/AP-5 into the VTA of rats reduced cocaine seeking elicited by cues in rats. Other studies have also reported that CNQX systemically administered (i.p.) modulated cocaine-induced SA or reinstatement to cocaine seeking. These data clearly state the participation of the glutamatergic neurotransmission through AMPA receptors in different processes related to cocaine-induced motivation and cocaine-seeking behaviour.

Our results also show that 100% of the SN animals extinguished the cocaine SA behaviour, while only 65% of the MSEW mice achieved the extinction criteria. Our data show a decreased CREB expression in the NAc of SN mice following the acquisition of cocaine SA and an increased pCREB expression in the NAc of MSEW mice. Previous studies have demonstrated that CREB reduction in the NAc of rats facilitates the extinction of cocaine seeking in the SA paradigm, while increased CREB in the NAc enhances cocaine reinforcement in the same paradigm. Additionally, the upregulation of CREB activity in the NAc induced by stress caused a pro-depressive phenotype in mice. The increased CREB activity induces the expression of BDNF and dynorphin, two genes that contribute to depressive-related behaviours. In fact, dynorphin activates kappa-opioid receptors in VTA neurons, inhibiting dopaminergic activity, and contributing to anhedonia and the development of depressive symptoms. To this effect, Gustafsson et al (2008) showed that MS in rats induces an increased expression of dynorphin in the NAc. Additionally, prodynorphin gene disruption and kappa-opioid receptor antagonists induce a potentiation of the rewarding effects of cocaine in mice.

We therefore propose that SN mice showed reduced CREB in the NAc following repeated cocaine exposure to reduce its rewarding effects, as a protective mechanism to counteract the deleterious effects of the drug and facilitate the extinction of cocaine SA behaviour. Nevertheless, this compensatory mechanism was not developed in the MSEW mice probably due to CREB levels are too low to reveal a further decrease. We also propose that increased pCREB levels in the NAc of MSEW mice were due to enhanced glutamatergic activity, which may increase the expression of dynorphin and inhibit the release of dopamine from VTA. This inhibition may contribute to the development of depressive-like behaviour, higher acquisition rates, and the tolerance to the rewarding effects of cocaine in MSEW mice, which could explain why mice increased the cocaine consumption during the acquisition phase.

Evaluating the alterations in the VTA, our results show that MSEW mice expressed lower basal GluR2 levels and higher GluR1/GluR2 basal ratio in comparison with the SN group, in accordance with an enhanced excitability of VTA dopaminergic neurons. This is supported by previous studies in rodents, which showed that enhanced excitability of VTA dopaminergic neurons is associated with a higher vulnerability to cocaine SA, depressive-like behaviour, and cocaine addiction. Therefore, we suggest that MSEW mice display an enhanced excitability of VTA dopaminergic neurons, probably to compensate for the higher GABAergic inhibition from the NAc. This enhanced excitability could contribute to the depressive-like phenotype of MSEW mice and their higher vulnerability to cocaine SA.

Even though previous studies in rats have reported no changes or increased expression of GluR1 in the VTA following cocaine consumption, we observed a significant GluR1 level decrease after cocaine SA in the VTA. This alteration could represent a compensatory effect to attenuate dopamine release induced by the drug. Additionally, GluR1 increased in terms of acquisition levels and reached basal condition during cocaine reinstatement. It is well known that cocaine induces long-term potentiation in excitatory synapses in the VTA and such adaptations have a pivotal role in the incubation of cocaine craving and cocaine-seeking. Likewise, studies in rodents have shown that cocaine abstinence produces a stronger activation of dopamine pathways due to hyperactivity dopamine neurotransmission. On the basis of such data, we propose that during the extinction phase, GluR1 levels promote the incubation of cocaine craving, thus enhancing the plasticity in this brain area. In this way, activation of dopaminergic function in the VTA is facilitated resulting in a stronger cocaine-seeking during the reinstatement phase.

Finally, cocaine SA acquisition reduced the levels of CREB and pCREB in the VTA of SN and MSEW mice, whereas cocaine-primed reinstatement induced an increase of CREB and pCREB levels in the same brain structure. Such results are in accordance with previous studies showing that cocaine priming increased pCREB and induced reinstatement in cocaine-conditioned animals. Moreover, Kreibich et al (2009) showed that chronic stress exposure enhances cocaine rewarding effects, a process that seems to be mediated by CREB. Accordingly, our results show that ELS induced by MSEW reduced

CREB levels and increased the pCREB/CREB ratio in the VTA, which go hand in hand with higher cocaine intake during SA acquisition.

In summary, our results propose that MSEW enhances glutamatergic function in the NAc and increases the excitability of VTA dopaminergic neurons, causing greater vulnerability to depression and cocaine-seeking behaviour. Our study may shed light on how molecular mechanisms in ELS alter the brain regions involved in reward and how they could contribute to finding new therapeutic targets to treat CUD.

ACKNOWLEDGEMENTS

This study was supported by the Ministerio de Economia y Competitividad (SAF2016-75966-R-FEDER and FPU 15/02492 to M. A. L.), Ministerio de Sanidad (Retic-ISCIII, RD16/017/010, and Plan Nacional sobre Drogas 2018/007). A. C.-Z. received the CONACYT grant (276577) from the Mexican government.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

AC-Z and OV were responsible for the study concept and design. AC-Z, AMS, and MAL carried out the experimental studies. AC-Z and OV drafted the manuscript and took part in interpreting findings. All authors critically reviewed the content and approved the final version for publication.

DATA AVAILABILITY STATEMENT

Data from our article will be available after request to the corresponding author.

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REFERENCES

1. Hyman SM, Paliwal P, Chaplin TM, Mazure CM, Rounsaville BJ, Sinha R. Severity of childhood trauma is predictive of cocaine relapse outcomes in women but not men. *Drug Alcohol Depend*. 2008;92(1-3):208-216.
2. Ducci F, Roy A, Shen P-H, et al. Association of substance use disorders with childhood trauma but not African genetic heritage in an African American cohort. *Am J Psychiatry*. 2009;166:1031-1040.
3. Lippard ETC, Nemeroff CB. The devastating clinical consequences of child abuse and neglect: increased disease vulnerability and poor treatment response in mood disorders. *Am J Psychiatry*. 2020;177(1):20-36.
4. Anda RF, Felitti VJ, Bremner JD, et al. The enduring effects of abuse and related adverse experiences in childhood: a convergence of evidence from neurobiology and epidemiology. *Eur Arch Psychiatry Clin Neurosci*. 2006;256(3):174-186.
5. Bagley JR, Szumlinski KK, Kippin TE. Discovery of early life stress interacting and sex-specific quantitative trait loci impacting cocaine responsiveness. *Br J Pharmacol*. 2019;176(21):4159-4172.
6. Gracia-Rubio I, Moscoso-Castro M, Pozo OJ, Marcos J, Nadal R, Valverde O. Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2016;65:104-117.
7. Gracia-Rubio I, Martínez-Laorden E, Moscoso-Castro M, Milanés V, Laorden L, Valverde O. Maternal separation impairs cocaine-induced behavioural sensitization in adolescent mice. *PLoS ONE*. 2016;11(12):e0167483.
8. Castro-Zavala A, Martín-Sánchez A, Valverde O. Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice. *Eur Neuropsychopharmacol*. 2020;32:12-24.
9. Dixon CI, Walker SE, Swinny J, et al. Early-life stress influences acute and sensitized responses of adult mice to cocaine by interacting with GABA A 2 receptor expression. *Behav Pharmacol*. 2019;30(2 and 3-Spec Issue):272-281.
10. Enoch M-A. The role of early life stress as a predictor for alcohol and drug dependence. *Psychopharmacology (Berl)*. 2011;214(1):17-31.
11. Wagner FA, Anthony JC. From first drug use to drug dependence: developmental periods of risk for dependence upon marijuana, cocaine, and alcohol. *Neuropsychopharmacology*. 2002;26(4):479-488.
12. Gass JT, Olive MF. Glutamatergic substrates of drug addiction and alcoholism. *Biochem Pharmacol*. 2008;75:218-265.
13. Bowers MS, Chen BT, Bonci A. AMPA receptor synaptic plasticity induced by psychostimulants: the past, present, and therapeutic future. *Neuron*. 2010;67(1):11-24.
14. Tang W-X, Fasulo W, Mash D, Hemby S. Molecular profiling of midbrain dopamine regions in cocaine overdose victims. *J Neurochem*. 2003;85(4):911-924.
15. Hemby S, Tang W, Muly E, Kuhar M, Howell L, Mash D. Cocaine-induced alterations in nucleus accumbens ionotropic glutamate receptor subunits in human and non-human primates. *J Neurochem*. 2005;95(6):1785-1793.
16. Choi KH, Edwards S, Graham DL, et al. Reinforcement-related regulation of AMPA glutamate receptor subunits in the ventral tegmental area enhances motivation for cocaine. *J Neurosci*. 2011;31(21):7927-7937.
17. Back SE, Hartwell K, DeSantis SM, et al. Reactivity to laboratory stress provocation predicts relapse to cocaine. *Drug Alcohol Depend*. 2010;106(1):21-27.
18. McFarland K, Lapish C, Kalivas P. Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. *J Neurosci*. 2003;23(8):3531-3537.
19. Conrad KL, Tseng KY, Uejima JL, et al. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature*. 2008;454(7200):118-121.
20. Moffett M, Harley J, Francis D, Sanghani SP, Davis WI, Kuhar MJ. Maternal separation and handling affects cocaine self-administration in both the treated pups as adults and the dams. *J Pharmacol Exp Ther*. 2006;317(3):1210-1218.
21. Kikusui T, Faccidomo S, Miczek KA. Repeated maternal separation: Differences in cocaine-induced behavioral sensitization in adult male and female mice. *Psychopharmacology (Berl)*. 2005;178:202-210.
22. Goffer Y, Xu D, Eberle SE, et al. Calcium-permeable AMPA receptors in the nucleus accumbens regulate depression-like behaviors in the chronic neuropathic pain state. *J Neurosci*. 2013;33(48):19034-19044.
23. Martínez-Rivera A, Hao J, Tropea TF, et al. Enhancing VTA Ca v 1.3 L-type Ca2+channel activity promotes cocaine and mood-related behaviors via overlapping AMPA receptor mechanisms in the nucleus accumbens. *Mol Psychiatry*. 2017;22(12):1735-1745.
24. Chocyk A, Bobula B, Dudys D, et al. Early-life stress affects the structural and functional plasticity of the medial prefrontal cortex in adolescent rats. *Eur J Neurosci*. 2013;38(1):2089-2107.

25. Carlezon WA, Thome J, Olson VG, et al. Regulation of cocaine reward by CREB. *Science (80-)*. 1998;282(5397):2272-2275.
26. Riday T, Kosofsky B, Malanga C. The rewarding and locomotor-sensitizing effects of repeated cocaine administration are distinct and separable in mice. *Neuropharmacology*. 2012;62(4):1858-1866.
27. Mattson BJ, Bossert JM, Simmons DE, et al. Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats is enabled by enhanced activation of extracellular signal-related kinase, but not protein kinase A. *J Neurochem*. 2005;95(5):1481-1494.
28. Olson VG, Zabetian CP, Bolanos CA, et al. Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. *J Neurosci*. 2005;25(23):5553-5562.
29. Kreibich AS, Blendy JA. cAMP response element-binding protein is required for stress but not cocaine-induced reinstatement. *J Neurosci*. 2004;24(30):6686-6692.
30. Sachs B, Tran H, Folse E, Caron M. Brain-region-specific molecular responses to maternal separation and social defeat stress in mice. *Neuroscience*. 2018;373:122-136.
31. Romano-López A, Méndez-Díaz M, García García F, Regalado-Santiago C, Ruiz-Contreras AE, Prospéro-García O. Maternal separation and early stress cause long-lasting effects on dopaminergic and endocannabinergic systems and alters dendritic morphology in the nucleus accumbens and frontal cortex in rats. *Dev Neurobiol*. 2015;76(8):819-831.
32. Bissonette GB, Roesch MR. Development and function of the mid-brain dopamine system: what we know and what we need to. *Genes Brain Behav*. 2015;1-12.
33. Bannon MJ, Pruetz B, Manning-Bog AB, et al. Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. *Proc Natl Acad Sci U S A*. 2002;99(9):6382-6385.
34. Anier K, Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A. Maternal separation is associated with DNA methylation and behavioural changes in adult rats. *Eur Neuropsychopharmacol*. 2014;24(3):459-468.
35. Larsen K, Momeni J, Farajzadeh L, Callesen H, Bendixen C. Molecular characterization and analysis of the porcine NURR1 gene. *Biochim Open*. 2016;3:26-39.
36. Lynch WJ, Mangini LD, Taylor JR. Neonatal isolation stress potentiates cocaine seeking behavior in adult male and female rats. *Neuropsychopharmacology*. 2005;30(2):322-329.
37. Vialou V, Robison AJ, Laplant QC, et al. ΔFosB in brain reward circuits mediates resilience to stress and antidepressant responses. *Nat Neurosci*. 2010;13(6):745-752.
38. Boudreau AC, Wolf ME. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci*. 2005;25(40):9144-9151.
39. Ping A, Xi J, Prasad BM, Wang M-H, Kruzich PJ. Contributions of nucleus accumbens core and shell GluR1 containing AMPA receptors in AMPA- and cocaine-primed reinstatement of cocaine-seeking behavior. *Brain Res*. 2008;1215:173-182.
40. Suto N, Ecke LE, Wise RA. Control of within-binge cocaine-seeking by dopamine and glutamate in the core of nucleus accumbens. *Psychopharmacology (Berl)*. 2009;205(3):431-439.
41. Park W-K, Bari AA, Jey AR, et al. Cocaine administered into the medial prefrontal cortex reinstates cocaine-seeking behavior by increasing AMPA receptor-mediated glutamate transmission in the nucleus accumbens. *J Neurosci*. 2002;22(7):2916-2925.
42. Mahler SV, Smith RJ, Aston-Jones G. Interactions between VTA orexin and glutamate in cue-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl)*. 2013;226:687-698.
43. Bäckström P, Hyytiä P. Attenuation of cocaine-seeking behaviour by the AMPA/kainate receptor antagonist CNQX in rats. *Psychopharmacology (Berl)*. 2003;166(1):69-76.
44. Bäckström P, Hyytiä P. Ionotropic and metabotropic glutamate receptor antagonism attenuates cue-induced cocaine seeking. *Neuropsychopharmacology*. 2006;31:778-786.
45. Larson EB, Graham DL, Arzaga RR, et al. Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. *J Neurosci*. 2011;31(45):16447-16457.
46. Nestler EJ. Role of the brain's reward circuitry in depression: transcriptional mechanisms. *Int Rev Neurobiol*. 2015;124:151-170.
47. Gustafsson L, Oreland S, Hoffmann P, Nylander I. The impact of postnatal environment on opioid peptides in young and adult male Wistar rats. *Neuropeptides*. 2008;42(2):177-191.
48. McLaughlin JP, Marton-Popovici M, Chavkin C. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci*. 2003;23(13):5674-5683.
49. Lüscher C. Cocaine-evoked synaptic plasticity of excitatory transmission in the ventral tegmental area. *Cold Spring Harb Perspect Med*. 2013;3(13):a012013.
50. Kreibich AS, Briand L, Cleck JN, Ecke L, Rice KC, Blendy JA. Stress-induced potentiation of cocaine reward: a role for CRF R1 and CREB. *Neuropsychopharmacology*. 2009;34(12):2609-2617.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Castro-Zavala A, Martín-Sánchez A, Luján MÁ, Valverde O. Maternal separation increases cocaine intake through a mechanism involving plasticity in glutamate signalling. *Addiction Biology*. 2020;e12911. https://doi.org/10.1111/adb.12911

Article 2

Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice.

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Eur Neuropsychopharmacoly. 2020 Mar;32:12-24.

DOI: https://doi.org/10.1016/j.euroneuro.2019.12.112

European Neuropsychopharmacology (2020) 32, 12–24

www.elsevier.com/locate/euroneuro

Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice

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Received 8 July 2019; received in revised form 13 November 2019; accepted 13 December 2019

KEYWORDS
Cocaine
self-administration;
GluA1;
GluA2;
CREB;
pCREB;
Maternal separation

Abstract
Even though men are more likely to use drugs, women tend to progress faster from drug use to drug abuse, especially in the case of psychostimulants such as cocaine. Preclinical studies evaluating the differences in cocaine self-administration (SA) between sexes are contradictory. While some have shown no between-sex differences, others have reported female rodents to acquire higher percentages of cocaine SA criteria. Furthermore, early-life adversity is a risk factor for substance-use disorder and clinical evidence showed that women who have experienced childhood adversity are more likely to use drugs in comparison with males. However, the molecular differences between sexes as a consequence of early-life adversity or cocaine consumption have scarcely been explored. The aim of our study was to evaluate the differences in the expression of the GluA1, GluA2 subunits of AMPA receptors, pCREB and CREB in male and female mice exposed to maternal separation with early weaning (MSEW). Moreover, we evaluated the effects of cocaine SA in both sexes during adulthood, and the possible changes in GluA1, GluA2, pCREB and CREB expressions. Our results showed a higher acquisition percentage in females and an MSEW-induced increase in cocaine-seeking solely in males. Additionally, we observed sex differences in GluA1, GluA2, CREB and pCREB levels in the NAc and the VTA. The

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https://doi.org/10.1016/j.euroneuro.2019.12.112

present results displayed changes in molecules that play a crucial role in the regulation of the rewarding effects of cocaine, helping to elucidate the mechanisms involved in the progression from cocaine use to cocaine abuse in both females and males.

1. Introduction

According to the statistics of drug consumption by sex, men are more prone to psychostimulant use than women (UNODC, 2018). However, once women begin to consume drugs, they tend to progress faster from use to abuse, a phenomenon known as telescoping (Haas and Peters, 2000). This phenomenon is especially evident in the case of psychostimulants such as cocaine (Johnson et al., 2019; Swalve et al., 2016; Zlebnik, 2019). Clinical studies have reported that female cocaine abusers exhibit a shorter latency from the first use to abuse and more admissions to treatments than men, thus demonstrating that women evolve differently in terms of cocaine addiction (Haas and Peters, 2000). A recent study evaluating differences in crack cocaine users found that women had an increased severity of cocaine use than men (Sanvicente-Vieira et al., 2019), Moreover, women used cocaine in higher quantity and frequency than men, females reported more symptoms at low doses of cocaine and showed a higher percentage of cocaine dependency (Chen and Kandel, 2002). Experimental studies have also shown that female rats acquire cocaine self-administration (SA) behaviour (Lynch, 2008) at a quicker rate and higher percentage, performing a greater number of infusions and consuming more cocaine than males (Cummings et al., 2011; Davis et al., 2008; Johnson et al., 2019; Peterson et al., 2014). Moreover, female rats show more incentive motivation for cocaine than males (Algallal et al., 2019) and develop psychomotor sensitisation to cocaine (Hu and Becker, 2003; Van Haaren and Meyer, 1991). Nevertheless, other studies have reported different results. Whilst Caine et al. (2004) appreciated no between-sex differences, Swalve et al. (2016) reported male rats to acquire at a higher percentage and in fewer sessions. Nevertheless, Swalve et al. (2016) also observed that once acquisition criteria were met, females did consume more cocaine. It is well known that cocaine induces alterations in neuronal structural plasticity in the mesolimbic system and the molecular mechanisms regulating its function (Golden and Russo, 2012).

In the past few years, cocaine-induced changes in AMPA glutamate receptors (AMPAr) have been increasingly explored (Bowers et al., 2010; Lüscher, 2013; Pierce and Wolf, 2013; Stuber et al., 2010). AMPARs are made up of four subunits (GluA1-A4) and are normally assembled by GluA2 in complex with either GluA1 or GluA3 (Bowers et al., 2010). The GluA2-lacking receptors are calcium-permeable AMPARs which play an important role in synaptic regulation (Man, 2011). Additionally, GluA2-lacking AMPARs are regulators of behaviour related to cocaine addiction and mood disorders. (Goffer et al., 2013; Martinez-Rivera et al., 2017). Extended access cocaine SA in male rats increases the levels of GluA1 and GluA2-lacking AMPARs in the nucleus accumbens (NAc), thus contributing to seeking behaviour (Conrad et al., 2008; Kalivas, 2009; Pierce and Wolf, 2013). Another study reported an upregulation of GluA1 and GluA2 in the ventral tegmental area (VTA) following cocaine SA, with GluA1 overexpression leading to increased lever-pressing (Choi et al., 2011). Moreover, cocaine-evoked synaptic plasticity in the VTA induces an exchange of GluA2-containing receptors for GluA2-lacking receptors, thus potentiating the excitatory transmission and firing of dopamine neurons (Luscher, 2013; Lüscher, 2013).

There are several factors that increase the vulnerability to develop cocaine addiction, such as childhood adversity (UNODC, 2018). A reliable animal model which allows us to reproduce the effects of childhood adversity is maternal separation with early weaning (MSEW) (Gracia-Rubio et al., 2016b; Portero-Tresserra et al., 2018). Studies evaluating the effects of early-life adversity in both sexes have shown sexual dimorphism in the expression of GluA1 and GluA2 mRNA levels in the hippocampus and amygdala of male and females rats (Katsouli et al., 2014). In maternally separated male rats, we observed a lower level of GluA2 in the NAc in comparison with the controls, with no changes in GluA1, whilst their female counterparts showed neither GluA2 nor GluA1 alterations (Ganguly et al., 2019). As for the VTA, we did not find any studies evaluating sex differences in the composition of the AMPAr.

Taken together, such data suggest that MSEW may induce sex-specific molecular changes stimulating alterations in AMPAr subunit composition, which could modify the acquisition of cocaine SA behaviour and the progression from cocaine use to cocaine abuse. Thus, the aim of our study was to evaluate the effects of MSEW in male and female mice and the molecular consequences of early-life adversity. Moreover, we analysed whether MSEW may have different effects on males and females in the acquisition of cocaine SA and the changes induced by cocaine exposure in GluA1, GluA2, pCREB and CREB in the VTA and the NAc of both sexes.

2. Experimental procedures

2.1. Animals

Fourteen male and fourteen female CD1 adult mice aged 10 weeks used as breeders (Charles River, Barcelona, Spain) were received at our animal facility, UBIOMEX, PRBB. The animals were placed in pairs in standard cages in a temperature- (21 ± 1 °C) and humidity- (55% ± 10%) controlled room and subjected to a 12 h light/dark cycle with the lights on from 8:00 to 20:00 h and *ad libitum* access to food and water. Ten days later, the males were removed from the cages. After that, the pups were used for the cocaine SA. The total number of animals used for the SA was 159 mice. The experiments were carried out in accordance with the guidelines of the European Communities Directive 88/609/EEC regulating animal research. All procedures were approved by the local ethical committee

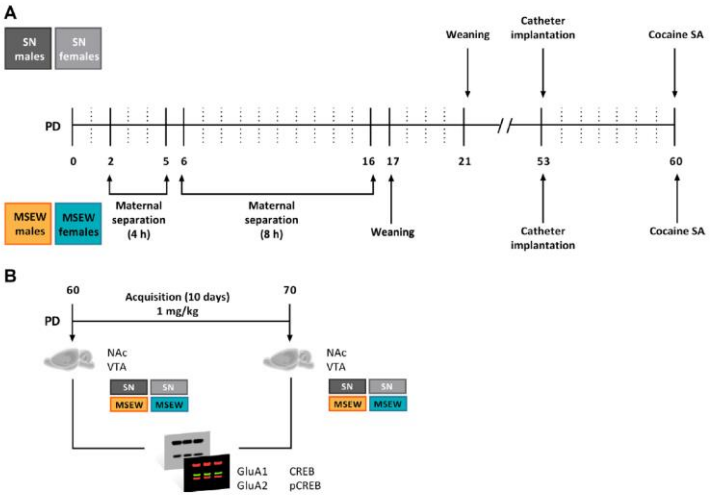

Fig. 1 Schematic representation of the experimental schedule. (A) Schematic representation of the MSEW model and the (B) timeline in which the brain samples were obtained.

(CEEA-PRBB) and every effort was made to minimise animal suffering and discomfort and to minimise the number of animals used. Briefly, when the MSEW (females $n = 25$, males $n = 57$) and SN (females $n = 27$, males $n = 50$).

2.2. Rearing conditions

The rearing conditions used were the same as previously described (Gracia-Rubio et al., 2016b; Portero-Tresserra et al., 2018) (Fig. 1(A)). Newborn mice were randomly assigned to the experimental groups: standard nest (SN) and MSEW. The day of birth was considered the postnatal day (PD) 0. Animals in the MSEW were separated from their mothers for 4 h per day (9:00 to 13:00 h) from PD2 to PD5, and 8 h per day (9:00 to 17:00 h) from PD6 to PD16. We have distributed the pups of each litter between the different experimental group in order to avoid a litter effect. We used less than 25% of the animals from the same litter. MSEW protocol does not affect body weight (Gracia-Rubio et al., 2016a; Portero-Tresserra et al., 2018), mortality (George et al., 2010), morbidity (George et al., 2010) or the male/female ratio (Koob and Zorrilla, 2010). For details see Supplementary Methods.

2.3. Drugs

Cocaine hydrochloride was purchased from Alcatel (Ministry of Health, Madrid, Spain) and was dissolved in sterile physiological saline (0.9%, NaCl solution). Cocaine was used at a dose of 1 mg/kg/infusion for the acquisition phase of the SA procedure.

2.4. Cocaine self-administration

The SA experiments were conducted as described (Ferrer-Pérez et al., 2019; Luján et al., 2018). Briefly, when the MSEW (females $n = 25$, males $n = 57$) and SN (females $n = 27$, males $n = 50$) animals reached PD53, jugular-vein catheter implantation was performed. The surgery for the intravenous catheter implantation was performed following anaesthetisation with a mixture of ketamine/xylazine (50 mg/mL, 10 mg/mL, administrated in a volume of 0.15 mL/10 g) animals were implanted with the jugular catheter. Animals were treated with analgesic (Meloxicam 0.5 mg/kg; i.p., administrated in a volume of 0.10 mL/10g) and antibiotic solution (Enrofloxacin 7.5 mg/kg; i.p., administrated in a volume of 0.03 mL/10 g). After surgery, animals were housed individually, placed over electric blankets, and allowed to recover. At least 3 days after surgery, the animals were trained, on a fixed ratio 1, to self-administer cocaine (1.0 mg/kg per infusion) for 10 daily sessions (2 h) and the number of infusions in the active and inactive holes was registered. Mice were considered to have acquired stable SA behaviour when the following criteria were met on 2 consecutive days: $\geq$5 responses on the active hole and $\geq$ 65% of responses on the active hole. The animals accomplished the 10 daily sessions independently of the day of acquisition (mean of acquisition day MSEW-females 7.17, MSEW-males 5.93, SN-females 4.78 and SN-males 5.10).

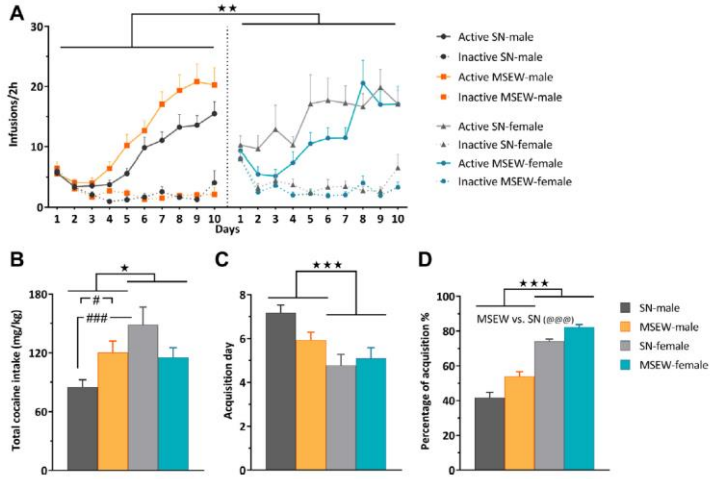

Fig. 2 Effects of MSEW on the acquisition of cocaine SA behaviour. Mean of infusions (A) in the active/inactive hole during the 10-day acquisition phase. Mean of total cocaine intake (B) during the acquisition phase. Day of acquisition (C) and percentage of animals (D) reaching acquisition criteria (SN-males n=23/56; MSEW-males n=30/55; SN-females n=23/27; MSEW-females n=20/28). *Sex* main effect of the ANOVA ($\star p<0.05$, $\star\star p<0.01$, $\star\star\star p<0.001$). *Rearing* main effect of the ANOVA (@@@$p<0.001$). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines ($\#p<0.05$, $\#\#\#p<0.01$). Data are expressed as mean ± SEM.

Only the mice achieving acquisition criteria were considered for the study: MSEW-females (n = 20), MSEW-males (n = 30), SN-females (n = 23) and SN-males (n = 23).

2.5. Animal sacrifice and sample collection

Animals were sacrificed by cervical dislocation and the brains were immediately removed from the skull and placed in a cold plaque. Brain samples were dissected at different times: drug-naïve and drug-experienced (30 min after the final acquisition day) (Fig. 1(B)). The drug-experienced animals are only animals that acquired cocaine SA behaviour. VTA and NAc were dissected and were immediately stored at −80 °C until the western blot assay was carried out.

2.6. Western blot for GluA2, GluA1, CREB and pCREB

To evaluate the expression of GluA2, GluA1, CREB and pCREB, samples were homogenised in cold lysis buffer (Table S1), supplemented with protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). Protein samples (16 μg) were mixed with 5X loading buffer (Table S2), loaded and run on SDS-PAGE 10% and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with BSA 5% for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies (Table S3). Primary antibodies were detected with fluorescent secondary antibodies (Table S4), incubated for 1 h at room temperature. Images were acquired on a Licor Odyssey Scanner and quantified using Image Studio Lite software v5.2 (LICOR, USA). The expression of GluA2, GluA1, CREB, pCREB and β-tubulin was evaluated in the VTA and NAc of the different groups: SN drug-naïve males, SN drug-experienced males, MSEW drug-naïve males, MSEW drug-experienced males, SN drug-naïve females, SN drug-experienced females, MSEW drug-naïve females and MSEW drug-experienced females (n = 5 per group, run in duplicate or triplicate). Firstly, we analysed the levels of GluA2, GluA1, CREB and pCREB (in drug-naïve animals), only normalising to β-tubulin (optical density, OD). Subsequently, data were normalised to the control group (SN drug-naïve) of each sex in order to ascertain the fold change due to MSEW, cocaine exposure or the combination of both variables.

2.7. Statistical analysis

Data were analysed for conditions of normality (Kolmogorov-Smirnov's test), sphericity (Mauchly's test) and homoscedasticity (Levene's test). Data for infusions were analysed by means of a

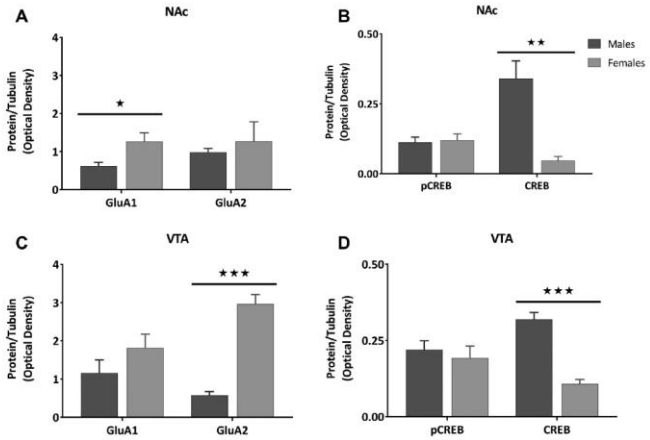

Fig. 3 Representation of the optical density relative to control protein tubulin in drug-naïve mice. (A) Mean of GluA1, GluA2 and mean of (B) pCREB and CREB in the NAc. (C) Mean of GluA1, GluA2 and mean of (D) pCREB and CREB in the VTA. *Sex* effect (⋆$p<0.05$, ⋆⋆$p<0.01$, ⋆⋆⋆$p<0.01$). Data are expressed as mean ± SEM (n=5, run in duplicate or triplicate).

four-way repeated measures ANOVA based on the following factors: *days*, *hole* (active or inactive), *sex* and *rearing*. Data for acquisition day, total cocaine intake and acquisition percentage were analysed using two-way ANOVA with *sex* and *rearing* as inter-subject variables. For western blot results, the OD of drug-naïve animals was analysed using a student's *t* test using *rearing* as a variable. Subsequently, the fold change of each molecule was analysed using a three-way ANOVA with *rearing*, *sex* and *phase* as independent factors. When F achieved $p < 0.05$, the ANOVA was followed by the Bonferroni post-hoc test if a main effect and/or interaction was observed. All possible post-hoc comparisons were evaluated. All statistical analyses were performed using SPSS Statistics v23. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered to be significant.

3. Results

3.1. MSEW potentiates cocaine SA and seeking behaviour in male mice, but not in female mice

The four-way ANOVA for the infusions (Fig. 2(A)) showed significant main effects of *days* ($F_{9,828} = 17.822$, $p < 0.001$), *hole* ($F_{1,92} = 190.581$, $p < 0.001$), *sex* ($F_{1,92}$=8.817, $p < 0.01$) and significant interactions: *days* × *hole* ($F_{9,828} = 33.420$, $p < 0.001$), *sex* × *rearing* ($F_{1,92} = 8.356$, $p < 0.01$), *sex* × *rearing* × *hole* ($F_{1,92} = 5.485$, $p < 0.05$) and *days* × *hole* × *rearing* ($F_{9,828} = 1.862$, $p < 0.05$). Bonferroni *post-hoc* analysis for the *sex* × *rearing* interaction revealed that SN-females performed a higher number of infusions than SN-males ($p < 0.05$). Moreover, the post-hoc analysis showed that MSEW-males carried out more infusions than SN-males ($p < 0.05$). In contrast, the registered data indicated that MSEW-females conducted fewer infusions than SN-females ($p < 0.001$). The *post-hoc* for the *sex* × *rearing* × *hole* interaction showed MSEW-males performing higher active infusions than SN-males ($p < 0.05$) and SN-females more active infusions than SN-males ($p < 0.01$). Moreover, the *post-hoc* analysis revealed that all groups were able to discriminate between holes (all cases $p < 0.001$). The interaction *days* × *hole* × *rearing* indicates that SN mice discriminated between holes from day 2 to the end of the acquisition while MSEW mice began to discriminate from day 4 to day 10.

3.2. Sex differences in the acquisition of cocaine SA behaviour and cocaine intake

For total cocaine intake (Fig. 2(B)), a two-way ANOVA was performed. The test showed a main effect of *sex* ($F_{1,92} = 5.432$, $p < 0.05$) and the interaction between factors *sex* × *rearing* ($F_{1,92} = 7.509$, $p < 0.01$). The *post-hoc* analysis showed that the SN-females consumed a greater amount of cocaine when compared to the SN-males ($p < 0.01$). Additionally, MSEW-males showed a higher cocaine intake than the SN-males ($p < 0.05$).

The two-way ANOVA for the day of acquisition (Fig. 2(C)) presented a main effect of *sex* ($F_{1,92} = 14.520$, $p < 0.001$), thus revealing that the females acquired cocaine SA behaviour earlier than the male mice.

The percentage of mice acquiring cocaine SA behaviour was 41.6% (SN-males, $n = 23/56$), 53.8% (MSEW-males, $n = 30/55$), 74.2% (SN-females, $n = 20/27$) and 82.3% (MSEW-females, $n = 23/28$) (Fig. 2(D)). Two-way ANOVA showed a main effect of *sex* ($F_{1,8} = 349.138$, $p < 0.001$) and *rearing* ($F_{1,8} = 38.819$, $p < 0.001$). The *sex* effect revealed a greater percentage of acquisition in the female mice compared to the males ($p < 0.001$). The *rearing* effect showed that MSEW increased the percentage of animals acquiring SA behaviour ($p < 0.001$).

3.3. Drug-naïve females show increased levels of GluA1 in NAc, GluA2 in the VTA and decreased CREB in both areas

In order to evaluate differences between sexes in the expression of GluA1, GluA2, pCREB and CREB, we analysed the OD of images obtained from western blot analysis of drug-naïve animals. Student's *t*-test for these proteins in the NAc showed the females to have higher GluA1 (Fig. 3(A)) ($t_8 = 5.031$, $p < 0.05$) and less CREB (Fig. 3(B)) levels than the males ($t_8 = 8.602$, $p < 0.05$). OD data from the VTA revealed that the females had higher GluA2 levels (Fig. 3(C)) ($t_8 = 5.504$, $p < 0.001$) and lower CREB expression (Fig. 3(D)) ($t_8 = 8.602$, $p < 0.05$) when compared to their male counterparts.

3.4. MSEW increases GluA1 fold change and the GluA1/GluA2 in the NAc of male mice, but not in females

Three-way ANOVA for the fold change of GluA1 revealed a *sex* × *rearing* interaction ($F_{1,32} = 4.027$, $p < 0.05$) (Fig. 4(A)). The Bonferroni *post-hoc* test revealed that MSEW-males showed higher GluA1 expression than SN-males ($p < 0.05$) and MSEW-females ($p < 0.05$). Three-way ANOVA for GluA2 fold change showed no significant changes (Fig. 4(B)).

The analysis for GluA1/GluA2 showed the main effect of *rearing* ($F_{1,32} = 4.659$, $p < 0.05$) and the interaction *sex* × *rearing* ($F_{1,32} = 8.880$, $p < 0.01$) (Fig. 4(C)). The *post-hoc* test for the *sex* × *rearing* interaction showed a higher ratio for the MSEW-males compared to the SN-males ($p < 0.01$) and MSEW-females ($p < 0.01$).

3.5. Cocaine SA and MSEW modify the GluA1/GluA2 ratio and the GluA1 fold change in the VTA of males

As for the GluA1 fold change, the three-way ANOVA displayed a main effect of *sex* ($F_{1,31}$=14.194, p<0.01) and the interaction *treatment* × *sex* ($F_{1,31}$=4.861, p<0.05) (Fig. 4(D)). The *post-hoc* analysis for the interaction *treatment* × *sex* showed that the acquisition of cocaine SA behaviour decreased the GluA1 fold change in male mice when compared to drug-naïve animals (p<0.05). The analysis also showed that, following the acquisition, females showed a higher GluA1 fold change than drug-experienced males (p<0.001).

Three-way ANOVA for GluA2 fold change in the VTA showed a main effect of *rearing* ($F_{1,31}$=5.955, p<0.05), *treatment* ($F_{1,31}$=4.566, p<0.05), *sex* ($F_{1,31}$=20.172, p<0.001), and the interactions *sex* × *rearing* ($F_{1,31}$=5.887, p<0.05) and *sex* × *rearing* × *treatment* ($F_{1,31}$=15.378, p<0.001) (Fig. 4(E)). In the Bonferroni *post-hoc* for the triple interaction, at drug-naïve MSEW-males showed a lower GluA2 fold change compared with the SN-males (p<0.001). Additionally, MSEW-males registered increased GluA2 fold change after acquisition (p<0.001) compared to drug-naïve MSEW-males.

The three-way ANOVA for the ratio GluA1/GluA2 displayed a *treatment* effect ($F_{1,31}$=21.177, p<0.001), and the interactions *rearing* × *treatment* ($F_{1,31}$=4.322, p<0.05), *sex* × *rearing* ($F_{1,31}$=8.723, p<0.01) and *treatment* × *sex* ($F_{1,31}$=20.391, p<0.001) (Fig. 4(F)). In the *post-hoc* for the *sex* × *rearing* interaction, the MSEW-males registered a higher ratio than the SN-males (p<0.01). The *post-hoc* test for *treatment* × *sex* showed that chronic exposition to cocaine during the acquisition phase decreased the GluA1/GluA2 ratio in the male mice when compared to the drug-naïve males (p<0.001). Furthermore, this interaction showed that drug-naïve males have a higher ratio than the drug-naïve females (p<0.01). Finally, in the *post-hoc* test for GluA1/GluA2, the female mice registered a higher ratio than the males (p<0.01), following acquisition.

3.6. The ratio pCREB/CREB increases in the NAc of self-administering male mice, but not in female mice

Three-way ANOVA for the pCREB fold change showed no significant changes (Fig. 5(A)). Three-way ANOVA for the CREB fold change revealed a main effect of *sex* ($F_{1,32}$=4.967, p<0.05), showing an increased CREB expression in females (Fig. 5(B)). The three-way ANOVA analysis for pCREB/CREB showed the interactions *rearing* × *treatment* ($F_{1,32}$=4.793, p<0.05) and *treatment* × *sex* ($F_{1,32}$=4.725, p<0.05) (Fig. 5(C)). In the *post-hoc* test for the *treatment* × *sex* interaction, the males registered a higher pCREB/CREB ratio than females (p<0.05), following acquisition.

3.7. Female mice display a higher fold change of CREB-phosphorylation and pCREB/CREB ratio than males in the VTA

Three-way ANOVA for the pCREB fold change showed a main effect of *treatment* effect ($F_{1,31}$=14.870, p<0.01), *sex* ($F_{1,31}$=28.538, p<0.001) and the interaction *treatment* × *sex* ($F_{1,31}$=7.323, p<0.05) (Fig. 5(D)). The Bonferroni *post-hoc* test for the interaction showed cocaine SA acquisition to decrease the pCREB fold change in males compared to drug-naïve mice (p<0.001), but also to increase pCREB fold change in females compared to males (p<0.001).

Three-way ANOVA for CREB fold change revealed a main effect of *rearing* ($F_{1,31}$=4.516, p<0.05) and

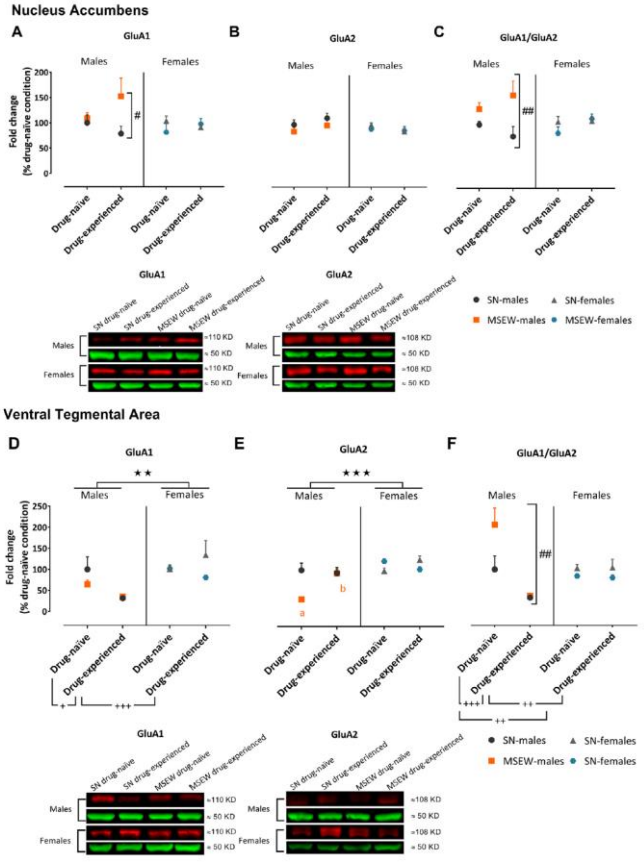

Fig. 4 Fold change of GluA1, GluA2 and GluA1/GluA2 in the NAc and VTA of SN and MSEW mice. Mean fold change relative to the control of (A) GluA1, (B) GluA2 and (C) GluA1/GluA2 levels in the NAc. Mean fold change relative to control of (D) GluA1, (E) GluA2 and (F) GluA1/GluA2 levels in the VTA. *Sex* main effect of the ANOVA (••$p<0.01$; •••$p<0.001$), *sex* × *rearing* interaction of the ANOVA (#$p<0.05$, ##$p<0.01$), *treatment* × *sex* interaction of the ANOVA (+$p<0.05$, ++$p<0.01$, +++$p<0.001$). Bonferroni *post-hoc* comparison for the triple interaction: (a) MSEW-males drug-naïve vs. SN-males drug-naïve ($p<0.001$); (b) MSEW-males drug-experienced vs. MSEW-males drug-naïve ($p<0.001$). The protein of interest in red and tubulin in green. Data are expressed as mean ± SEM (n=5, run in duplicate or triplicate). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

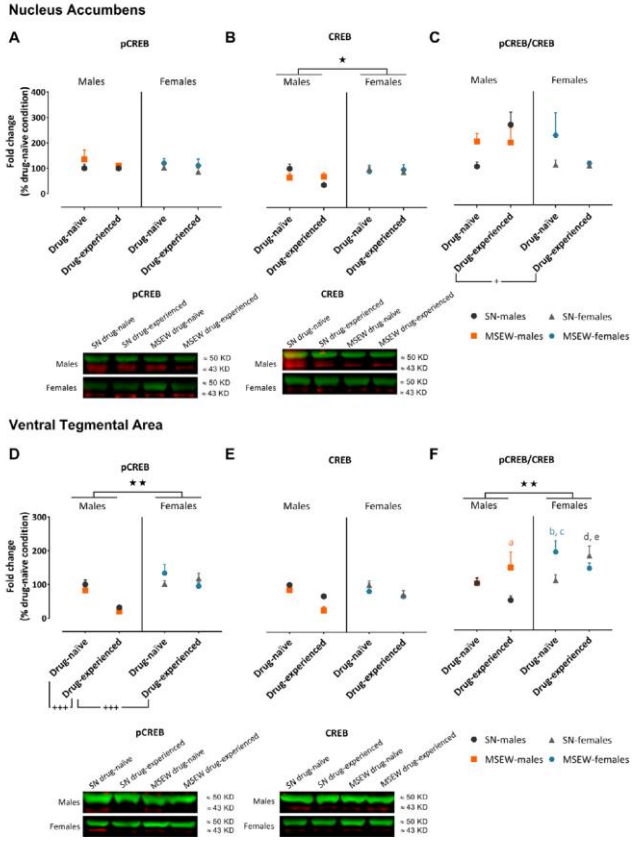

Fig. 5 Fold change of pCREB, CREB and pCREB/CREB in the NAc and the VTA of SN and MSEW mice. Mean fold change relative to the control of (A) pCREB, (B) CREB and (C) pCREB/CREB levels in the NAc. Mean fold change relative to control of (D) pCREB, (E) CREB and (F) pCREB/CREB levels in the VTA. *Sex* main effect of the ANOVA ($\star p<0.05$, $\star\star p<0.01$), *treatment* × *sex* interaction of the ANOVA ($+p<0.05$, $+++p<0.001$). Bonferroni *post-hoc* comparison for the triple interaction: (a) MSEW-males drug-experienced vs. SN-males drug-experienced ($p<0.05$), (b) MSEW-females drug-naïve vs. MSEW-males drug-naïve ($p<0.05$); (c) MSEW-females drug-naïve vs. SN-females drug-naïve ($p<0.05$); (d) SN-females drug-experienced vs. SN-males drug-experienced ($p<0.01$); (e) SN-females drug-experienced vs. SN-females drug-naïve ($p<0.05$). The protein of interest in red and tubulin in green. Data are expressed as mean ± SEM ($n=5$, run in duplicate or triplicate). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment ($F_{1,31}$=13.308, p<0.01) (Fig. 5(E)). The *rearing* effect showed MSEW to decrease the CREB fold change (p<0.05) and the *treatment* effect revealed that the acquisition of cocaine SA behaviour decreased the CREB fold change in mice (p<0.01).

For the ratio pCREB/CREB, three-way ANOVA displayed a *sex* effect ($F_{1,31}$=10.086, p<0.01) and the interaction *sex* × *rearing* × *treatment* ($F_{1,31}$=8.938, p<0.01) (Fig. 5(F)). The *post-hoc* analysis for the triple interaction revealed that, following acquisition, MSEW-males showed a higher pCREB/CREB ratio than the SN-males (p<0.05). We also observed that MSEW increased the ratio pCREB/CREB in drug-naïve females, in comparison with SN-females (p<0.05) and MSEW-males (p<0.05). Furthermore, the results showed cocaine SA acquisition to increase the pCREB/CREB ratio only in SN-females (p<0.05). Finally, in the *post-hoc* analysis, self-administering SN-females registered a higher pCREB/CREB ratio than self-administering SN-males (p<0.01).

4. Discussion

In our study, the reinforcing effects of cocaine were more powerful in the female mice compared to their male counterparts in the SA paradigm. Our results show that MSEW-mice increased the percentage of acquisition (males and females together) compared with SN-mice. Nevertheless, females were not affected by the MSEW in the number of infusions, total cocaine intake or the day of task acquisition. Additionally, our study revealed that MSEW enhances cocaine-seeking behaviour and cocaine intake, albeit solely in male mice.

Western blot results displayed sex-specific changes induced by MSEW in the expression of AMPA receptor subunits in the NAc and VTA and also for the CREB expression in both areas. To our knowledge, this is the first study to explore sex-specific AMPA-mechanism changes in mice induced by cocaine SA in the VTA and NAc.

Epidemiologic studies have shown that women are more vulnerable to develop drug addiction due to the telescoping effect (Haas and Peters, 2000; Lynch et al., 2002; UNODC, 2018). Lynch et al. (2002) reported women to be more susceptible to cocaine use than men. Preclinical studies have shown that females have higher reinforcing effects of cocaine in the SA (Caine et al., 2004; Cummings et al., 2011; Davis et al., 2008; Johnson et al., 2019; Lynch, 2008; Peterson et al., 2014). In accordance with such results, we observed that female mice showed a faster and higher percentage of SA behaviour acquisition, in addition to greater cocaine-seeking and intake, thus displaying a different progression and higher vulnerability to cocaine addiction.

It is known that childhood adversity is a risk factor to develop drug addiction (Haas and Peters, 2000; UNODC, 2018). Clinical studies have shown that childhood adversity affects men and women in different ways. Elton et al. (2014) showed sex-dependent effects of childhood adversity on the functional and effective regulation of brain inhibitory network. Animal studies evaluating the effects of neonatal isolation in rats reported higher cocaine consumption in the females, independently of the isolation procedure, and also the tendency of isolated males to consume more cocaine than the control males (Kosten et al., 2007, 2004; Lynch et al., 2005). A recent study has shown that social isolation stress potentiates motivation and leads to increased infusions during cocaine SA only in male mice (Newman et al., 2018). In our study, we also observed that the effect of early-life stress-induced by MSEW is sex-dependent. Males were negatively affected by the MSEW, while females appeared to be more resilient to this early-life stress, as previously demonstrated (Kikusui et al., 2005). A recent study showed that continuous social defeat stress increased or attenuated cocaine SA in male mice (Arena et al., 2019). Moreover, they observed that social stress divides the mice in groups (high and low responders), indicating that social stress contributes to developing different reward-seeking phenotype dependent of the individual response to stress experience and that this could be led by changes in neural mechanisms that will induce vulnerability or resilience to the stress experience (Arena et al., 2019).

Several studies have reported that cocaine exposure induces long-term potentiation (LTP) in the VTA and NAc (Hemby et al., 2005; Nestler, 2001). LTP induction triggers the exocytosis of perisynaptic AMPARs in the membrane in order to induce a transient activation of the NMDA receptors (Andrásfalvy et al., 2004). These new AMPARs contain GluA1 subunits (GluA2-lacking AMPARs) (Yang et al., 2010, 2008) and are responsible for the full expression of the LTP (Yang et al., 2008). Once inserted, the subunit GluA1 is rapidly changed by GluA2 to stabilise the new synapse (Adesnik and Nicoll, 2007; Yang et al., 2008). Evidence has shown that cocaine exposure induces the translocation of GluA2 for GluA1 in the NAc, and that a cocaine-induced GluA1increase contributes to seeking behaviour (Conrad et al., 2008; Kalivas, 2009; Pierce and Wolf, 2013). In accordance with such results, we observed that animals with increased GluA1 expression in the NAc (females) showed increased cocaine seeking (Fig. 3(A)). We hypothesise that this increased GluA1 in the drug-naïve females allows them to generate a faster and stronger cocaine-induced LTP, thus explaining why females acquire cocaine SA behaviour earlier than males and why they registered a higher percentage of acquisition.

Our results also show that MSEW-males, but no females, registered a positive GluA1 fold change and a higher GluA1/GluA2 ratio in NAc. Such molecular alterations may explain why only males exposed to this early-life stress registered an increase in the number of infusions and cocaine intake during SA. Moreover, the MSEW-induced male-specific increase of GluA1/GluA2 may enhance the excitability of NAc neurons, which would lead to a higher GABAergic inhibition of VTA dopaminergic neurons.

Our results also show that males expressed higher CREB (pCREB/CREB) activation than females after cocaine SA in the NAc. Previous studies evaluating the effect of social defeat stress in male rats showed that this kind of stress decreased pCREB in the NAc (Yap et al., 2015). Even our results showed no significant effect of the MSEW, we observed that maternally separated mice tended to show increased pCREB/CREB ratio, meaning that MSEW increases the activation of CREB. Sustained elevations of CREB activity in the NAc produces an anhedonia-like profile

(Barrot et al., 2002), being in accordance with our data. One neuroadaptation reported to compensate cocaine-induced dopamine release is the activation of CREB in the GABAergic neurons of the NAc, which induce dynorphin expression (Muschamp and Carlezon, 2013). CREB-mediated dynorphin augmentation reduces dopamine transmission, inhibiting κ opioid receptor (KOR) activity in the VTA and NAc (Muschamp and Carlezon, 2013). We therefore suggest that, after chronic cocaine exposure, males increase their pCREB/CREB ratio as a compensatory mechanism to avoid the negative effects of the drug. Like cocaine, chronic stress also activates the CREB pathway promoting depressive like-behaviour (Covington et al., 2011). In agreement with this statement, we observe that MSEW upregulates CREB activation (pCREB/CREB) in the NAc of both sexes, in accordance with our earlier observation that MSEW induces depressive-like behaviour (Gracia-Rubio et al., 2016b). In line with our results, Fosnocht et al. (2019) observed that social isolation stress during adolescence, increases responding for cocaine in male mice but not in females, but also that cocaine exposure after adolescent stress increases c-Fos expression in the NAc independently of the sex. Therefore, they suggest that this increased behavioural responsivity to cocaine could be regulated by the glutamatergic system (Fosnocht et al., 2019).

In addition to the alterations in the NAc, we evaluated changes in the VTA, which play a key role in the reinforcing effects of cocaine (Nestler, 2001). Cocaine-evoked VTA synaptic plasticity is mediated by changes in the glutamatergic synapses and the new protein synthesis is triggered by cocaine-induced LTP (Heshmati, 2009). Some studies have reported that cocaine SA increases GluA1 and GluA2 in rats and that GluA1 overexpression in the VTA enhances level-press behaviour in the same paradigm (Choi et al., 2011). Our results show that MSEW increased the GluA1/GluA2 ratio in the VTA of males, but not in females. Furthermore, MSEW drug-naïve males show lower GluA2 than the SN drug-naïve males and even lower than the MSEW drug-naïve females (Fig. 4(E)). We would therefore suggest that males, especially those exposed to MSEW, showed enhanced excitability of the VTA dopaminergic neurons, most probably to compensate the higher GABAergic inhibition from the NAc. This male-specific increased function of the AMPAr may explain why MSEW only affects the acquisition of cocaine SA in males. Our results revealed that cocaine SA acquisition induced a negative fold change of GluA1 and GluA1/GluA2 ratio in VTA in males, whereas the GluA1 fold change and GluA1/GluA2 ratio in females, after cocaine exposure, were unaffected.

In this way, GluA1 transcription is regulated by CREB (Olson et al., 2005), and CREB activation modulates the rewarding effects of cocaine in the VTA (Tang et al., 2003). Moreover, tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, is another target gene for CREB in the VTA (Nestler, 2001; Olson et al., 2005). Accordingly, our results show that drug-experienced males registered lower pCREB and, consequently, GluA1 expressions.

On the basis of such results, we hypothesised that, as males show a higher basal excitability of VTA neurons (GluA1/GluA2), cocaine consumption would induce a higher dopamine release in males than in females, being in agreement with the results of Holly et al. (2012) in which male rats exposed to social stress showed a greater change from baseline dopamine levels in response to cocaine, in comparison with non-stressed males. However, between stressed and non-stressed females, no statistical difference was found. Subsequently, as a compensatory mechanism, males showed decreased CREB activation (pCREB), GluA1 expression, and possibly tyrosine hydroxylase synthesis, and finally dopamine firing. Normally, VTA dopaminergic projections to the prefrontal cortex, inhibit glutamatergic prefrontal cortex neurons (Pirot et al., 1992). Therefore, reduced dopamine firing in the VTA implies greater glutamatergic activity from the prefrontal cortex to the NAc. The NAc-glutamatergic hyperactivation increases pCREB, thus inducing the dynorphin synthesis to inhibit the release of dopamine in the VTA. As stated earlier, only males showed increased pCREB in the NAc, possibly to induce dynorphin synthesis and amortise the cocaine-induced dopamine release in the VTA. Therefore, the dysphoric effects in males would seem to be stronger than in females, which may be considered a protective measure against cocaine addiction. Such a hypothesis is supported by a recent study showing females to be less sensitive than males to the KOR-mediated reward-decreasing effect, due to higher VTA tyrosine hydroxylase levels, which increase dopamine synthesis and protect them against the suppression of dopamine release and anhedonia (Conway et al., 2019). This hypothesis is also in agreement with the results showing that tonic dopamine levels in the NAc were not different due to sex or social stress factors. However, after cocaine exposure (i.p), stressed animals showed important changes of dopamine levels in NAc compared with the non-stressed rats, but cocaine-induced dopamine elevation lasted longer in females (Holly et al., 2012).

In summary, our results support the idea that being a female is a risk factor to develop cocaine addiction. Our results also suggest that, although MSEW appears to be a risk factor in cocaine addiction, it affects males to a greater degree, as females seem to be more resilient to this kind of early-life stress. A possible explanation is that MSEW enhances excitability in the NAc of males, potentiating the GABAergic inhibition. However, as drug-naïve females showed a higher GluA1 level, they were less affected by the MSEW-induced alteration. Therefore, the higher GluA1 level in females would seem to explain why they are more vulnerable to cocaine addiction but resilient to such stress. Furthermore, males exhibit higher excitability of dopaminergic neurons in the VTA, especially in the case of MSEW-exposed males (Fig. 4(C)). However, after chronic cocaine exposure, the higher excitability decreased in relation to the levels of the drug-naïve animals. Therefore, males would seem to show stronger cocaine-induced dysphoric effects, which could indeed be a protective factor in the escalation of cocaine use. Another possible interpretation for the lack of differences in the cocaine SA paradigm between MSEW and SN females, is the presence of a ceiling effect, and hence the cocaine intake in the SA could not be further potentiated by the MSEW. Regardless of which interpretation is correct, this study yields novel insight about sex and maternal neglect differences in cocaine-seeking behaviour.

Role of the funding source

This study was supported by the Ministerio de Economia y Competitividad (grant number SAF2016-75966-R-FEDER), Ministerio de Sanidad, Asuntos Sociales e Igualdad (Retic-ISCIII, RD16/017/010 and Plan Nacional sobre Drogas 2018/007). A.C-Z received Consejo Nacional de Ciencia y Tecnologia (CONACYT, grant number 276577) from the Mexican government.

Contributors

A.C.Z. and O.V. were responsible for the study concept and design. A.C.Z. and A.M.S. carried out the experimental studies. A.C.Z and O.V. drafted the manuscript and participated in the interpretation of findings. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank Gerald-Patrick Fannon for his English proofreading and editing of the manuscript.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.12.112.

References

Adesnik, H., Nicoll, R.A., 2007. Conservation of glutamate receptor 2-containing AMPA receptors during long-term potentiation. J. Neurosci. 27, 4598-4602. doi:10.1523/JNEUROSCI.0325-07.2007.

Algallal, H., Allain, F., Ndiaye, N.A., Samaha, A., 2019. Sex differences in cocaine self-administration behaviour under long access versus intermittent access conditions. Addict. Biol. doi:10.1111/adb.12809.

Andrásfalvy, B.K., Magee, J.C., Andrásfalvy, B.K., 2004. Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones. J. Physiol. 559, 543-554. doi:10.1113/jphysiol.2004.065219.

Arena, D.T., Covington, H.E., DeBold, J.F., Miczek, K.A., 2019. Persistent increase of I.V. cocaine self-administration in a subgroup of C57BL/6J male mice after social defeat stress. Psychopharmacology 236, 2027-2037. doi:10.1007/s00213-019-05191-6.

Barrot, M., Olivier, J.D.A., Perrotti, L.I., DiLeone, R.J., Berton, O., Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C., Zachariou, V., Nestler, E.J., 2002. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc. Natl. Acad. Sci. 99, 11435-11440. doi:10.1073/pnas.172091899.

Bowers, M.S., Chen, B.T., Bonci, A., 2010. AMPA receptor synaptic plasticity induced by psychostimulants: the past, present, and therapeutic future. Neuron 67, 11-24. doi:10.1016/j.neuron.2010.06.004.

Caine, S.B., Bowen, C.A., Yu, G., Zuzga, D., Negus, S.S., Mello, N.K., 2004. Effect of gonadectomy and gonadal hormone replacement on cocaine self-administration in female and male rats. Neuropsychopharmacology 29, 929-942. doi:10.1038/sj.npp.1300387.

Chen, K., Kandel, D., 2002. Relationship between extent of cocaine use and dependence among adolescents and adults in the United States. Drug Alcohol Depend. 68, 65-85. doi:10.1016/S0376-8716(02)00086-8.

Choi, K.H., Edwards, S., Graham, D.L., Larson, E.B., Whisler, K.N., Simmons, D., Friedman, A.K., Walsh, J.J., Rahman, Z., Monteggia, L.M., Eisch, A.J., Neve, R.L., Nestler, E.J., Han, M.-H., Self, D.W., 2011. Reinforcement-related regulation of AMPA glutamate receptor subunits in the ventral tegmental area enhances motivation for cocaine. J. Neurosci. 31, 7927-7937. doi:10.1523/JNEUROSCI.6014-10.2011.

Conrad, K.L., Tseng, K.Y., Uejima, J.L., Reimers, J.M., Heng, L.-J., Shaham, Y., Marinelli, M., Wolf, M.E., 2008. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature 454, 118-121. doi:10.1038/nature06995.

Conway, S.M., Puttick, D., Russell, S., Potter, D., Roitman, M.F., Chartoff, E.H., 2019. Females are less sensitive than males to the motivational- and dopamine-suppressing effects of kappa opioid receptor activation. Neuropharmacology 146, 231-241. doi:10.1016/j.neuropharm.2018.12.002.

Covington, H.E., Maze, I., Sun, H., Bomze, H.M., DeMaio, K.D., Wu, E.Y., Dietz, D.M., Lobo, M.K., Ghose, S., Mouzon, E., Neve, R.L., Tamminga, C.A., Nestler, E.J., 2011. A role for repressive histone methylation in cocaine-induced vulnerability to stress. Neuron 71, 656-670. doi:10.1016/j.neuron.2011.06.007.

Cummings, J.A., Gowl, B.A., Westenbroek, C., Clinton, S.M., Akil, H., Becker, J.B., 2011. Effects of a selectively bred novelty-seeking phenotype on the motivation to take cocaine in male and female rats. Biol. Sex Differ. 2, 3. doi:10.1186/2042-6410-2-3.

Davis, B.A., Clinton, S.M., Akil, H., Becker, J.B., 2008. The effects of novelty-seeking phenotypes and sex differences on acquisition of cocaine self-administration in selectively bred high-responder and low-responder rats. Pharmacol. Biochem. Behav. 90, 331-338. doi:10.1016/j.pbb.2008.03.008.

Elton, A., Tripathi, S.P., Mletzko, T., Young, J., Cisler, J.M., James, G.A., Kilts, C.D., 2014. Childhood maltreatment is associated with a sex-dependent functional reorganization of a brain inhibitory control network. Hum. Brain Mapp. 35, 1654-1667. doi:10.1002/hbm.22280.

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.

Fosnocht, A.Q., Lucerne, K.E., Ellis, A.S., Olimpo, N.A., Briand, L.A., 2019. Adolescent social isolation increases cocaine seeking in male and female mice. Behav. Brain Res. 359, 589-596. doi:10.1016/j.bbr.2018.10.007.

Ganguly, P., Honeycutt, J.A., Rowe, J.R., Demaestri, C., Brenhouse, H.C., 2019. Effects of early life stress on cocaine conditioning and AMPA receptor composition are sex-specific and driven by TNF. Brain Behav. Immun. doi:10.1016/j.bbi.2019.01.006.

George, E.D., Bordner, K.A., Elwafi, H.M., Simen, A.A., 2010. Maternal separation with early weaning: a novel mouse model of early life neglect. BMC Neurosci. 11, 123. doi:10.1186/1471-2202-11-123.

Goffer, Y., Xu, D., Eberle, S.E., D'amour, J., Lee, M., Tukey, D., Froemke, R.C., Ziff, E.B., Wang, J., 2013. Calcium-

permeable AMPA receptors in the nucleus accumbens regulate depression-like behaviors in the chronic neuropathic pain state. J. Neurosci. 33, 19034-19044. doi:10.1523/JNEUROSCI.2454-13.2013.

Golden, S.A., Russo, S.J., 2012. Mechanisms of psychostimulant-induced structural plasticity. Cold Spring Harb. Perspect. Med. 2. doi:10.1101/cshperspect.a011957.

Gracia-Rubio, I., Martinez-Laorden, E., Moscoso-Castro, M., Milanés, V., Laorden, L., Valverde, O., 2016a. Maternal separation impairs cocaine-induced behavioural sensitization in adolescent mice. PLoS One 11, e0167483. doi:10.1371/journal.pone.0167483.

Gracia-Rubio, I., Moscoso-Castro, M., Pozo, O.J., Marcos, J., Nadal, R., Valverde, O., 2016b. Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 65, 104-117. doi:10.1016/j.pnpbp.2015.09.003.

Haas, A.L., Peters, R.H., 2000. Development of substance abuse problems among drug-involved offenders: evidence for the telescoping effect. J. Subst. Abuse 12, 241-253. doi:10.1016/S0899-3289(00)00053-5.

Hemby, S., Tang, W., Muly, E., Kuhar, M., Howell, L., Mash, D., 2005. Cocaine-induced alterations in nucleus accumbens ionotropic glutamate receptor subunits in human and non-human primates. J. Neurochem. 95, 1785-1793. doi:10.1111/j.1471-4159.2005.03517.x.

Heshmati, M., 2009. Cocaine-induced LTP in the ventral tegmental area: new insights into mechanism and time course illuminate the cellular substrates of addiction. J. Neurophysiol. 101, 2735-2737. doi:10.1152/jn.00127.2009.

Holly, E.N., Shimamoto, A., Debold, J.F., Miczek, K.A., 2012. Sex differences in behavioral and neural cross-sensitization and escalated cocaine taking as a result of episodic social defeat stress in rats. Psychopharmacology 224, 179-188. doi:10.1007/s00213-012-2846-2.

Hu, M., Becker, J.B., 2003. Effects of sex and estrogen on behavioral sensitization to cocaine in rats. J. Neurosci. 23, 693-699.

Johnson, A.R., Thibeault, K.C., Lopez, A.J., Peck, E.G., Sands, L.P., Sanders, C.M., Kutlu, M.G., Calipari, E.S., Kutlu, G., Calipari, E.S., 2019. Cues play a critical role in estrous cycle-dependent enhancement of cocaine reinforcement. Neuropsychopharmacology 1. doi:10.1038/s41386-019-0320-0.

Kalivas, P.W., 2009. The glutamate homeostasis hypothesis of addiction. Nat. Rev. Neurosci. 10, 561-572. doi:10.1038/nrn2515.

Katsouli, S., Stamatakis, A., Giompres, P., Kouvelas, E.D., Stylianopoulou, F., Mitsacos, A., 2014. Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain. Neuroscience 257, 49-64. doi:10.1016/j.neuroscience.2013.10.073.

Kikusui, T., Faccidomo, S., Miczek, K.A., 2005. Repeated maternal separation: differences in cocaine-induced behavioral sensitization in adult male and female mice. Psychopharmacology 178, 202-210. doi:10.1007/s00213-004-1989-1.

Koob, G.F., Zorrilla, E.P., 2010. Neurobiological mechanisms of addiction: focus on corticotropin-releasing factor. Curr. Opin. Investig. Drugs 11, 63-71.

Kosten, T.A., Karanian, D.A., Yeh, J., Haile, C.N., Kim, J.J., Kehoe, P., Bahr, B.A., 2007. Memory impairments and hippocampal modifications in adult rats with neonatal isolation stress experience. Neurobiol. Learn. Mem. 88, 167-176. doi:10.1016/j.nlm.2007.03.011.

Kosten, T.A., Sanchez, H., Zhang, X.Y., Kehoe, P., 2004. Neonatal isolation enhances acquisition of cocaine self-administration and food responding in female rats. Behav. Brain Res. 151, 137-149. doi:10.1016/j.bbr.2003.08.010.

Luján, M.Á., Castro-Zavala, A., Alegre-Zurano, L., Valverde, O., 2018. Repeated Cannabidiol treatment reduces cocaine intake and modulates neural proliferation and CB1R expression in the mouse hippocampus. Neuropharmacology 143, 163-175. doi:10.1016/j.neuropharm.2018.09.043.

Luscher, C., 2013. Drug-evoked synaptic plasticity causing addictive behavior. J. Neurosci. 33, 17641-17646. doi:10.1523/JNEUROSCI.3406-13.2013.

Lüscher, C., 2013. Cocaine-evoked synaptic plasticity of excitatory transmission in the ventral tegmental area. Cold Spring Harb. Perspect. Med. 3, a012013. doi:10.1101/cshperspect.a012013.

Lynch, W., Roth, M., Carroll, M., 2002. Biological basis of sex differences in drug abuse: preclinical and clinical studies. Psychopharmacology 164, 121-137. doi:10.1007/s00213-002-1183-2.

Lynch, W.J., 2008. Acquisition and maintenance of cocaine self-administration in adolescent rats: effects of sex and gonadal hormones. Psychopharmacology 197, 237-246. doi:10.1007/s00213-007-1028-0.

Lynch, W.J., Mangini, L.D., Taylor, J.R., 2005. Neonatal isolation stress potentiates cocaine seeking behavior in adult male and female rats. Neuropsychopharmacology 30, 322-329. doi:10.1038/sj.npp.1300594.

Man, H.-Y., 2011. GluA2-lacking, calcium-permeable AMPA receptors-inducers of plasticity? Curr. Opin. Neurobiol. 21, 291-298. doi:10.1016/j.conb.2011.01.001.

Martinez-Rivera, A., Hao, J., Tropea, T.F., Giordano, T.P., Kosovsky, M., Rice, R.C., Lee, A., Huganir, R.L., Striessnig, J., Addy, N.A., Han, S., Rajadhyaksha, A.M., 2017. Enhancing VTA Ca v 1.3 L-type Ca2+channel activity promotes cocaine and mood-related behaviors via overlapping AMPA receptor mechanisms in the nucleus accumbens. Mol. Psychiatry 22, 1735-1745. doi:10.1038/mp.2017.9.

Muschamp, J.W., Carlezon, W.A., 2013. Roles of nucleus accumbens CREB and dynorphin in dysregulation of motivation. Cold Spring Harb. Perspect. Med. 3 a012005-a012005. doi:10.1101/cshperspect.a012005.

Nestler, E.J., 2001. Molecular basis of long-term plasticity underlying addiction. Nat. Rev. Neurosci. 2, 119-128. doi:10.1038/35053570.

Newman, E.L., Leonard, M.Z., Arena, D.T., de Almeida, R.M.M., Miczek, K.A., 2018. Social defeat stress and escalation of cocaine and alcohol consumption: Focus on CRF. Neurobiol. Stress 9, 151-165. doi:10.1016/j.ynstr.2018.09.007.

Olson, V.G., Zabetian, C.P., Bolanos, C.A., Edwards, S., Barrot, M., Eisch, A.J., Hughes, T., Self, D.W., Neve, R.L., Nestler, E.J., 2005. Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. J. Neurosci. 25, 5553-5562. doi:10.1523/JNEUROSCI.0345-05.2005.

Peterson, A.B., Hivick, D.P., Lynch, W.J., 2014. Dose-dependent effectiveness of wheel running to attenuate cocaine-seeking: Impact of sex and estrous cycle in rats. Psychopharmacology 231, 2661-2670. doi:10.1007/s00213-014-3437-1.

Pierce, R.C., Wolf, M.E., 2013. Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. Cold Spring Harb. Perspect. Med. 3, a012021. doi:10.1101/cshperspect.a012021.

Pirot, S., Godbout, R., Mantz, J., Tassin, J.P., Glowinski, J., Thierry, A.M., 1992. Inhibitory effects of ventral tegmental area stimulation on the activity of prefrontal cortical neurons: evidence for the involvement of both dopaminergic and GABAergic components. Neuroscience 49, 857-865. doi:10.1016/0306-4522(92)90362-6.

Portero-Tresserra, M., Gracia-Rubio, I., Cantacorps, L., Pozo, O.J., Gómez-Gómez, A., Pastor, A., López-Arnau, R., de la Torre, R., Valverde, O., 2018. Maternal separation increases alcohol-drinking behaviour and reduces endocannabinoid levels in the mouse striatum and prefrontal cortex. Eur. Neuropsychopharmacol. 28, 499-512. doi:10.1016/j.euroneuro.2018.02.003.

Sanvicente-Vieira, B., Rovaris, D.L., Ornell, F., Sordi, A., Rothmann, L.M., Niederauer, J.P.O., Schuch, J.B., von Diemen, L., Kessler, F.H.P., Grassi-Oliveira, R., 2019. Sex-based differences in multidimensional clinical assessments of early-abstinence crack cocaine users. PLoS One 14. doi:10.1371/journal.pone.0218334.

Stuber, G.D., Hopf, F.W., Tye, K.M., Chen, B.T., Bonci, A., 2010. Neuroplastic alterations in the limbic system following cocaine or alcohol exposure. Curr. Top. Behav. Neurosci. 3, 3-27. doi:10.1007/7854_2009_23.

Swalve, N., Smethells, J.R., Carroll, M.E., 2016. Sex differences in the acquisition and maintenance of cocaine and nicotine self-administration in rats. Psychopharmacology 233, 1005-1013. doi:10.1007/s00213-015-4183-8.

Tang, W.-X., Fasulo, W., Mash, D., Hemby, S., 2003. Molecular profiling of midbrain dopamine regions in cocaine overdose victims. J. Neurochem. 85, 911-924. doi:10.1046/j.1471-4159.2003.01740.x.

United Nation Office on Drugs and Crime (UNODC). World Drug Report 2018.

Van Haaren, F., Meyer, M.E., 1991. Sex differences in locomotor activity after acute and chronic cocaine administration. Pharmacol. Biochem. Behav. 39, 923-927. doi:10.1016/0091-3057(91)90054-6.

Yang, Y., Wang, X.-b., Frerking, M., Zhou, Q., 2008. Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long-term potentiation. Proc. Natl. Acad. Sci. 105, 11388-11393. doi:10.1073/pnas.0802978105.

Yang, Y., Wang, X.-b., Zhou, Q., 2010. Perisynaptic GluR2-lacking AMPA receptors control the reversibility of synaptic and spines modifications. Proc. Natl. Acad. Sci. doi:10.1073/pnas.0913004107.

Yap, J.J., Chartoff, E.H., Holly, E.N., Potter, D.N., Carlezon, W.A., Miczek, K.A., 2015. Social defeat stress-induced sensitization and escalated cocaine self-administration: the role of ERK signaling in the rat ventral tegmental area. Psychopharmacology 232, 1555-1569. doi:10.1007/s00213-014-3796-7.

Zlebnik, N.E., 2019. Females pay a higher price for addiction. Neuropsychopharmacology 44, 1179-1181. doi:10.1038/s41386-019-0373-0.

Article 3

Cocaine-induced impulsivity is differentially expressed in male and female mice exposed to maternal separation and is associated with alterations in AMPA receptors subunits.

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DOI: https://doi.org/10.1101/2020.06.05.136812

Cocaine-induced impulsivity is differentially expressed in male and female mice exposed to maternal separation and is associated with alterations in AMPA receptors subunits.

Running title: Cocaine-induced impulsivity in male and female mice

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ABBREVIATIONS

AMPArs: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

CSF: Lumbar cerebrospinal fluid

GABA: Gamma-aminobutyric acid

GluA1: AMPA receptor subunit 1

GluA2: AMPA receptor subunit 2

Gria1: Glutamate Ionotropic Receptor AMPA Type Subunit 1 gen

Gria2: Glutamate Ionotropic Receptor AMPA Type Subunit 2 gen

MDD: Major depression disorder

NAc: Nucleus accumbens

mPFC: medial prefrontal cortex

MSEW: Maternal separation with early weaning

PD: Postnatal day

PFC: Prefrontal cortex

RRID: Research Resource Identifier

SA: Self-administration

SN: Standard nest

SUD: Substance use disorder

TST: Tail suspension test

VTA: Ventral tegmental area

ABSTRACT

Impulsivity is a key trait in the diagnosis of major depressive disorder (MDD) and substance use disorder (SUD). MDD is a chronic illness characterized by sadness, insomnia, and loss of interest. SUD is a chronic and relapsing disorder characterized by the consumption of drugs despite their negative consequences. Among drugs of abuse, cocaine is the most consumed psychostimulant. Animal studies demonstrated that increased impulsivity predicts predisposition to acquire cocaine self-administration (SA) behaviour with an increased cocaine-intake. Moreover, early-life stress represents a vulnerability factor to develop depressive disorders and drug addiction. Maternal separation with early weaning (MSEW) is an animal model that allows examining the impact of early-life stress on cocaine abuse. In this study, we aimed to explore changes in MSEW-induced impulsivity to determine potential associations between depression-like and cocaine-seeking behaviours in male and female mice. We also evaluated possible alterations in the AMPA receptors (AMPARs) composition and glutamatergic neurotransmission. We exposed mice to MSEW and the behavioural tests were performed during adulthood. Moreover, GluA1, GluA2 mRNA and protein expression were evaluated in the medial Prefrontal Cortex (mPFC). Results showed higher impulsive cocaine-seeking in females, independently the MSEW, as well as an increase in GluA1 and GluA2 protein expression. Moreover, MSEW induced downregulation of Gria2 and increased the GluA1/GluA2 ratio, only in male mice. In conclusion, female mice expressed higher mPFC glutamatergic function, which potentiated their impulsivity during cocaine SA. Also, data indicated that MSEW alters glutamatergic

function in mPFC of male mice, increasing the glutamatergic excitability.

Keywords: cocaine self-administration, depression-like behaviour, Gria1, Gria2, GluA1, GluA2

INTRODUCTION

Impulsivity is a major personality and temperament dimension consisting of maladaptive behaviour and characterized by poorly conceived, prematurely expressed, unduly risky, or inappropriate actions often resulting in undesirable consequences (Granö et al. 2007; Dent and Isles 2014; Dalley and Ersche 2019). Therefore, this personality trait can increase predisposition to suffer drug addiction (Nicholls et al. 2014; Rømer Thomsen et al. 2018; Adams et al. 2019; Butelman et al. 2019; Jupp et al. 2020) and other psychiatric disorders like major depressive disorder (MDD) (Dent and Isles 2014; Dalley and Ersche 2019). Depression is the most common psychological disorder affecting more than 264 million people worldwide (James et al. 2018). This disorder is characterized by sadness, loss of interest or pleasure, feelings of guilt, low self-worth, disturbed sleep or appetite, tiredness, and poor concentration (WHO 2017),

Impulsivity has been also proposed as an endophenotype to investigate the underlying neurobiological mechanisms for many impulse control disorders including substance use disorder (SUD) (Dalley and Ersche 2019). Among illicit drugs, cocaine is one of the most consumed psychostimulants with more than 18 million users (UNODC 2019). Clinical studies show that cocaine addiction includes poor inhibitory control for goal-directed behaviour in the frontal cortical regions, evidencing an impulse control disorder which induces drug craving (Barrós-Loscertales et al. 2019). In line with this, preclinical studies demonstrated that increased impulsivity predicts predisposition for drug use to evolve towards drug abuse (Jupp et al. 2020).

Animal studies show that rats with high impulsivity met the acquisition criterion faster, in greater percentage and also consumed more cocaine than rats with low impulsivity (Perry et al. 2005). In females rats, it was observed that impulsivity could predict the levels of acquisition of cocaine self-administration (SA) (Perry et al. 2005). Moreover, in male rats, high impulsivity predicts the development of compulsive cocaine use (Belin et al. 2008). Additionally, in a genetic animal model of impulsivity, Roman high-avoidance rats showed increased cocaine sensitization (Giorgi et al. 2005), higher number of responses in the SA paradigm (Fattore et al. 2009) and higher vulnerability to self-administer cocaine (Fattore et al. 2009). All of these behavioural alterations were associated with decreased volume and function of some mesocorticolimbic areas related with the development and persistence of cocaine addiction (Fattore et al. 2009; Giorgi et al. 2019; Río-Álamos et al. 2019).

Several studies have also demonstrated that cocaine exposure induces changes in the glutamatergic system, including the AMPA receptors (AMPArs) subunit composition (Bowers et al. 2010; Castro-Zavala et al. 2020a; Castro-Zavala et al. 2020b). AMPArs are made up of four subunit proteins (GluA1-A4) and generally composed of GluA2 in complex with GluA1 or GluA3 (Bowers et al. 2010). Therefore, previous works showed increased AMPArs function after cocaine exposure, because of the induction of long-term potentiation (Kauer and Malenka 2007). This high activity of AMPArs could be explained by the insertion of GluA2-lacking AMPArs, because this kind of receptors are calcium-permeable, have greater channel conductance and trigger calcium-dependent signalling cascades (Bowers et al. 2010).

Regarding impulsivity and the vulnerability to cocaine abuse, Nakamura et al (2000) showed that the administration of NBQX, an AMPArs antagonist, decreased impulsivity in a dose-dependently way in rats. Additionally, Barkus et al. (2012) making use of a GluA1 knock-out mice model with an impulsive phenotype, reported a faster acquisition in the food SA paradigm and a decreased capacity to extinguish the SA behaviour. These studies evidenced the key role played by the AMPArs in the modulation of impulsivity.

As previously mentioned, there is also a close relationship between impulsivity and MDD. In fact, GluA2-lacking AMPArs were suggested to be a common link between depression and SUD (Goffer et al. 2013; Martínez-Rivera et al. 2017; Castro-Zavala et al. 2020b). Clinical studies reported increased impulsivity in adults with a later MDD diagnosis, suggesting impulsivity as a predictor of the development of major depression (Granö et al. 2007). Also, Corruble et al. (2003) described three characteristics of impulsivity in adults with severe depression: behavioural loss of control, non-planned activities and cognitive impulsivity. In line with these studies, some authors have evaluated the association between impulsivity and childhood adversity in depressed adults (Brodsky et al. 2001). These studies reported that those patients with history of childhood trauma showed higher impulsivity and higher suicidal behaviour than the ones without childhood adversity (Brodsky et al. 2001).

Maternal separation with early weaning (MSEW) is an animal model that allows researchers to reproduce the effects of childhood adversity (George et al. 2010; Vetulani 2013; Bian et al. 2015). Moreover, MSEW induces a depression phenotype that permits the examination of the

impact of early-life stress on cocaine use or abuse (Liu et al. 2018; Vannan et al. 2018; Castro-Zavala et al., 2020b). We have previously reported, using a model of MSEW, an increased depression- and anxiety-like behaviour(Gracia-Rubio et al. 2016b; Portero-Tresserra et al. 2018), higher acquisition in the cocaine SA paradigm in males but not in females (Castro-Zavala et al. 2020b) and increased GluA1/GluA2 in the NAc and VTA of males exposed to MSEW and cocaine SA (Castro-Zavala et al. 2020b). Moreover, we also observed an increased acquisition percentage in females, which was independent of the early-life stress (Castro-Zavala et al. 2020b), being in accordance with the telescoping effect observed in women regarding drug use disorders (Haas and Peters 2000).

In this context, this work aimed to explore the changes in impulsivity induced by the MSEW model in males and females CD1 mice. Moreover, we determined a possible association between depression-like behaviour, impulsive cocaine-seeking, as well as alterations of AMPArs subunit (mRNA and protein levels). To achieve these goals, we evaluated impulsivity for cocaine intake (as a percentage of response efficiency) in the SA paradigm. We also determined GluA1 and GluA2 protein expression and evaluated Gria1 and Gria2 mRNA expression in the medial prefrontal cortex (mPFC), a brain area involved in inhibitory control of behaviours.

MATERIALS AND METHODS

Animals

Sixteen male and sixteen female CD1 adult mice of 10 weeks of age were used as breeders (Charles River, Barcelona, Spain). Animals were received at our animal facility, UBIOMEX, PRBB. The animals were placed in pairs in standard cages at a temperature- (21 ± 1ºC) and humidity- (55% ± 10%) controlled room and subjected to a 12 h light/dark cycle; with the lights on from 8:00 to 20:00 h and *ad libitum* access to food and water. Ten days later, the males were removed from the cages. Once offspring had been weaned, mice were assigned randomly to the cocaine SA, food SA or to naïve condition. A different group of mice was used to perform the tail suspension test (TST). The total number of animals used for this work was 189. Experimenters were blinded to the different experimental procedures. The experiments were carried out in accordance with the guidelines of the European Communities Directive 88/609/EEC regulating animal research. The local ethical committee (CEEA-PRBB) approved all procedures, and every effort was made to minimize animal suffering and discomfort as well as the number of animals used.

Rearing conditions

The rearing conditions were as previously described (Gracia-Rubio et al. 2016b; Portero-Tresserra et al. 2018; Castro-Zavala et al. 2020a; Castro-Zavala et al. 2020b). New-born mice were randomly assigned to the experimental groups: standard nest (SN) and MSEW (Figure 1A). The day of birth was considered the postnatal day (PD) 0. Animals in

the MSEW were separated from their mothers for 4 h per day (9:00 to 13:00 h) from PD2 to PD5, and 8 h per day (9:00 to 17:00 h) from PD6 to PD16. As for the separation, the mother was removed and placed in another cage and room, leaving the pups in their home box. To maintain the body temperature of the pups, home boxes were placed upon electric blankets until the mother was duly returned. Animals in the SN remained with their mother until weaning (PD21), whilst animals in the MSEW were weaned at PD17. In both cases (SN and MSEW), cages were cleaned on PD10. We distributed the pups of each litter between the different experimental groups in order to avoid a litter effect. MSEW procedure does not affect body weight (Gracia-Rubio et al. 2016a; Portero-Tresserra et al. 2018), mortality (George et al. 2010), morbidity (George et al. 2010) or the male/female ratio (Koob and Zorrilla 2010).

Tail suspension test

Mice underwent the TST on PD60 as previously described (Gracia-Rubio et al. 2016b). Briefly, each mouse was suspended 50 cm above a benchtop for 6 minutes (using adhesive tape attached 1cm from the tip of the tail). The time (s) that the animal was immobile during this interval was recorded.

Drugs

Cocaine hydrochloride was purchased from Alcatel (Ministry of Health, Madrid, Spain) and was dissolved in sterile physiological saline (0.9%, NaCl solution). A dose of 1 mg/kg/infusion was used for the acquisition phase of the SA procedure.

Apparatus for self-administration experiments

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 into the active hole produced a reinforcement (cocaine infusion or a food pellet) that was paired with two stimulus lights, one of which was placed inside the nose-poke and the other above the active hole. Mice received a maximum of 150 reinforcements, and each reinforcement was followed by a 15 s time-out period, in which no cocaine infusions were delivered. Nose-poking into the inactive hole had no consequences. The side on which the active/inactive hole was placed was counterbalanced.

At the beginning of each session, the house light was ON for 3 s and OFF for the rest of the experiment. The session started with a food pellet release or a cocaine priming injection and 4 s presentation of the light cue, situated above the active hole.

Cocaine self-administration

The SA experiments were conducted as described (Ferrer-Pérez et al. 2019; Castro-Zavala et al. 2020a; Castro-Zavala et al., 2020b). Briefly, when the SN (males n=23, females n=23) and MSEW (males n=30, females n=20) animals reached PD53, a jugular-vein catheter implantation was performed. The surgery was done following anaesthetization with a mixture of ketamine/xylazine (50 mg/mL, 10 mg/mL, administrated in a volume of 0.15 mL/10g). Animals were treated with analgesic (Meloxicam 0.5 mg/kg; i.p, administrated in a

volume of 0.10 mL/10g) and antibiotic solution (Enrofloxacin 7.5 mg/kg, i.p., administrated in a volume of 0.03 mL/10 g). After surgery, animals were housed individually, placed over electric blankets, and allowed to recover. At least 3 days after surgery, animals were trained, on a fixed ratio 1, to self-administer cocaine (1.0 mg/kg per infusion). During 10-day sessions (2 h each), the amount of nosepokes in the active- and the inactive hole (responses during time in and time out) and the infusion number (responses during time in) were counted. Mice were considered to have acquired a stable SA behaviour when the following criteria were met on 2 consecutive days: ≥5 responses in the active hole and ≥ 65% of responses in the active hole. All animals accomplished the 10 sessions independently of the day of acquisition.

Food self-administration

Four days before testing commenced (PD56), mice SN (males n=10, females n=18) and MSEW (males n=10, females n=12) were food-restricted and for that, mice were fed accordingly to the 95% of their body mass daily. Food restriction lasted the duration of food-maintained operant behaviour. Water was available *ad libitum* during the experimental phase. The animals were trained, on a fixed ratio 1, to nosepoke for food pellets (Grain-Based Rodent #5001, Test Diet, Sawbridgeworth, UK) for 10-day sessions (2 h each). The nosepoke number in the active- and the inactive hole (responses during time in and time out) and the pellet number (responses during time in) were counted.

Percentage of response efficiency and motor impulsivity

The evaluation of motor impulsivity could be divided into two processes: impulsive action and impulsive choice (Dalley et al. 2011; Dalley and Ersche 2019). Impulsive action is measured by the incapacity to self-restraint and perform anticipatory actions, known as a failure of motor inhibition (Dalley et al. 2011; Dalley and Ersche 2019).

Adapting the formula employed by Hynes et al. (2018) to our SA paradigm, we calculated the percentage of response efficiency as an indirect measure of impulsivity. A 100% response efficiency represents enough responses to obtain the reinforcement (one food pellet/one nosepoke or one cocaine infusion/one nosepoke). Therefore, decreased response efficiency means an increased impulsive response.

For the cocaine SA, we used the following formula:

$$\%\ Response\ efficiency = \frac{Number\ of\ cocaine\ infusions}{Number\ of\ active\ nosepokes} \times 100$$

In the case of food SA, we calculated the percentage of response efficiency as follows:

$$\%\ Response\ efficiency = \frac{Number\ of\ food\ pellets}{Number\ of\ active\ nosepokes} \times 100$$

As a result, mice with high percentage response efficiency scores were considered less impulsive, while mice with low percentage response efficiency were considered more impulsive (Hynes et al. 2018).

Animal Sacrifice and Sample Collection

Animals were sacrificed by cervical dislocation. Brains were immediately removed from the skull and placed in a cold plaque. Samples were dissected at different phases: without any behavioural test (drug-naïve), after cocaine SA (drug-experienced) and after TST (PD60). The drug-experienced animals are exclusively the mice that acquired the cocaine SA behaviour. mPFC was dissected and immediately stored at -80ºC until the biochemical analysis was performed. Samples from naïve- and drug-experienced mice were used for the western blot. For the qPCR, we utilised samples from mice that completed the TST.

Western Blot for GluA1 and GluA2

To evaluate the expression of GluA1 and GluA2, 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 a protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and a phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). Protein samples (20 μg) were mixed with 5X loading buffer (TRIS pH 6.8 0.153 M, SDS 7.5%, Glycerol 40%, EDTA 5 mM, 2-β-mercaptoethanol 0.025%, bromophenol blue 0.025%), loaded and run on SDS-PAGE 10% and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with BSA 5% for 1 h at room temperature and incubated overnight at 4ºC with primary antibodies (Table 1). Primary antibodies were detected with fluorescent secondary antibodies (Table 1), incubated for 1 h at room temperature. Images were acquired on a Licor Odyssey Scanner and quantified using Image Studio Lite

software v5.2 (LICOR, USA). The expression of GluA1, GluA2 and β-tubulin were evaluated in the mPFC of the different groups: SN drug-naïve males, SN drug-experienced males, MSEW drug-naïve males, MSEW drug-experienced males, SN drug-naïve females, SN drug-experienced females, MSEW drug-naïve females and MSEW drug-experienced females (n=4-5 per group, run in triplicate). Data were normalized to the SN naïve males in order to determine the fold change due to sex, MSEW, cocaine exposure or the interaction between variables.

RNA isolation and real-time (RT)-PCR

RNA extraction from mPFC samples was performed using trizol as previously described (Cardenas-Perez et al. 2018). RT-PCR was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using random primers and following standardized protocols.

Quantitative PCR for Gria1 and Gria2

For the qPCR, we use cDNA (20 ng), Light Cycler SBYR green 480 Master Mix (Roche LifeScience, Product No. 04707516001) and the specific primers (Table 2) for Gria1, Gria2 and 36B4 as housekeeping gene (Integrated DNA Techologies, Inc.). The qPCR was performed in LightCycler ® 480 Instrument II (Roche LifeScience) using next program: 95°C-10s, 60°C-20s, 72°C-10s for 45 cycles.

Statistical Analysis

Data were analysed for conditions of normality (Kolmogorov-Smirnov`s test), sphericity (Mauchly's test) and homoscedasticity (Levene's test). Data from the TST, qPCR, average of response efficiency and western blot results of drug-naïve mice were analysed using a two-way ANOVA with *rearing* and *sex* as independent factors. Western blot results of drug-naïve and drug-experienced animals were analysed using a three-way ANOVA with *rearing*, *sex* and *treatment* as factors. Data of percentage of response efficiency were analysed using a three-way ANOVA repeated measures with *rearing*, *sex* and *days* as factors of variation. When F achieved $p<0.05$, the ANOVA was followed by the Bonferroni post-hoc test if a main effect and/or interaction was observed. All possible post-hoc comparisons were evaluated. Statistical analyses were performed using SPSS Statistics v23. Data are expressed as mean ± SEM and a value of $p<0.05$ was considered significant.

RESULTS

Maternal separation increases depression-like behaviour only in male mice.

The effects observed in the TST were evaluated in female and male mice during adulthood (PD60) in MSEW and control mice (Figure 2). A two-way ANOVA of immobility time showed a significant effect of *sex* ($F_{1,39}=12.324$, $p<0.01$), *rearing* ($F_{1,39}=10.291$, $p<0.01$) and the interaction between these factors ($F_{1,39}=4.908$, $p<0.05$). The *sex* effect evidenced higher immobile time in females than males ($p<0.01$) while the *rearing*

effect showed that MSEW mice remained immobile for a longer time ($p<0.01$). Bonferroni post-hoc test for the interaction showed that SN females spent more time immobile than SN males ($p<0.001$) and also that MSEW males showed a higher immobile time than SN males ($p<0.01$).

MSEW downregulates Gria2 mRNA and increases the Gria1/Gria2 ratio in the mPFC of male mice.

We sought to identify the effect of MSEW on the gene expression of Gria1 and Gria2 and the possible relation between depression-like behaviour and the levels of these genes. For this purpose, we performed qPCR in the mPFC of males and females after the TST. For Gria1, we did not find significative differences (Figure 3A). Results for the Gria2 showed a significative main effect of *rearing* ($F_{1,36}=5.939$, $p<0.05$) and the interaction *sex* × *rearing* ($F_{1,36}=10.577$, $p<0.01$) (Figure 3A). The *rearing* effect showed that MSEW significantly decreased the transcription of Gria2 ($p<0.05$). The interaction *sex* × *rearing* showed a decreased Gria2 mRNA level in SN females in comparison with SN males ($p<0.01$). Moreover, the interaction also showed that maternally separated males had downregulation of Gria2 mRNA level in comparison with the SN males ($p<0.001$). For the Gria1/Gria2 ratio and the statistical analysis showed a significative difference for the interaction *sex* × *rearing* ($F_{1,36}=9.516$, $p<0.01$) (Figure 3A). Bonferroni post-hoc test revealed a significant increased Gria1/Gria2 ratio ($p<0.05$) in MSEW males ($p<0.01$) compared to the SN males. Additionally, the analysis showed that MSEW females had a decreased Gria1/Gria2 ratio than the MSEW males ($p<0.05$).

Depressive-like behaviour correlates with Gria2 mRNA levels in the mPFC of mice.

The relationship between change in Gria2 mRNA and the immobility time during the TST was determined using Pearson correlation coefficient. There was a significant correlation ($r=-0.4518$, $p<0.01$, $n=40$) between Gria2 and the depression-like behaviour among the subjects (Figure 3B). Linear regression suggests that Gria2 mRNA level could be predicted from the depression-like behaviour ($F_{1,38}=9.744$, $p<0.01$) ($y=-0.002244x+1.075$) (Figure 3C).

Increased GluA1 and GluA2 protein expression in female compared to male mice.

Because of the differences observed in the Gria1 and Gria2 mRNA of mice, we decided to evaluate whether these changes were also manifested in the functional form of these genes. Hence, we detected the protein expression of GluA1 (Figure 3D), GluA2 (Figure 3E) and the GluA1/GluA2 ratio (Figure 3F), using western blotting in male and female mPFC of drug-naïve mice. The two-way ANOVA showed a main *sex* effect for GluA1 ($F_{1,16}=8.006$, $p<0.05$) and GluA2 ($F_{1,16}=7.087$, $p<0.05$). In both cases, the sex effect evidenced that females showed an increased protein expression compared to males. No changes were found for the GluA1/GluA2 ratio.

Females show higher impulsive cocaine-seeking independently of the early-life stress.

In order to determine if depressed animals exposed to MSEW showed different impulsivity for cocaine, we performed the cocaine SA

procedure and calculated the average of the percentage of response efficiency along the acquisition phase (Figure 4A). The two-way ANOVA for the average of the response efficiency showed a main effect of *sex* ($F_{1,92}$=8.016, $p<0.01$) and the interaction *sex* × *rearing* ($F_{1,92}$=5.011, $p<0.05$). The *sex* effect indicated a lower response efficiency in female mice, meaning that females showed higher impulsivity to seek cocaine than males ($p<0.01$). The interaction *sex* × *rearing* revealed that maternally separated male mice had lower response efficiency than SN males ($p<0.01$), suggesting that MSEW increases impulsivity for cocaine-seeking but only in males. This interaction also evidenced that SN females showed higher cocaine-seeking impulsivity (decreased response efficiency) than SN males ($p<0.01$), behaving in the same way than the MSEW males and the MSEW females.

Cocaine but not natural reward SA is affected by early-life stress in male mice.

To evaluate if the MSEW-induced increased impulsivity was specific for cocaine, we performed a food SA in another set of animals. Once again, we calculated the average of the percentage of response efficiency along the acquisition phase (Figure 4B). The two-way ANOVA did not show *sex* effect, *rearing* effect or the interaction between these two factors, suggesting that changes in impulsivity induced by MSEW affected cocaine-related impulsivity rather than responses related to natural reward.

Chronic cocaine exposure does not modify the expression of GluA1, GluA2 or the GluA1/GluA2 ratio in the mPFC of mice.

In order to evaluate changes in the expression of GluA1 (Figure 5A), GluA2 (Figure 5B) or the GluA1/GluA2 ratio (Figure 5C) due to cocaine exposure, we determined the levels of these proteins in the mPFC of mice that accomplish the cocaine SA. We also compared the values of these animals with the values of the drug-naïve mice. Three-way ANOVA revealed a *sex* effect for GluA1 ($F_{1,32}$=31.094, $p<0.001$) and GluA2 ($F_{1,32}$=6.905, $p<0.05$). In both cases, this main effect showed that females had higher protein expression than males. Moreover, statistical analysis showed a *sex* × *rearing* interaction for GluA1. The Bonferroni post-hoc test for the interaction specified that: SN females showed a higher GluA1 level than the SN males ($p<0.05$), MSEW females, higher than MSEW male mice ($p<0.001$) and also that MSEW females had increased protein expression than the SN females ($p<0.05$). For the GluA1/GluA2 ratio we did not find any significative differences.

DISCUSSION

The present study shows that early-life stress induces a depressive-like behaviour in adult male mice whilst MSEW-exposed females seem to be resilient to this type of stress. Moreover, glutamatergic neurotransmission in the mPFC was differently affected due to rearing conditions and sex. Thus, Gria2 levels in the mPFC of MSEW male mice were different than in the SN males, showing that MSEW affected both sexes differently. However, in the case of GluA2, protein levels were not different between the SN males than to those exposed to early-

life stress (MSEW). As measured in the cocaine SA, female mice showed increased impulsivity for cocaine-seeking independently of the early-life stress exposure. However, males were significantly affected by the MSEW, which increased their impulsivity for cocaine-seeking. Moreover, we observed that the MSEW-increased impulsivity in males was specific for cocaine because all the groups showed similar percentages of response efficiency in the food SA. Results from western blot in drug-naïve animals showed that female mice expressed higher levels of GluA1 and GluA2 in the mPFC, which could explain why they manifest higher impulsivity to cocaine intake and resilience to early-life stress. In sum, our results show a sex-dependent effect in the impulsivity to cocaine consumption and this effect seems to be cocaine-specific.

In previous work, we have also reported that only males were negatively affected by the MSEW in the acquisition of cocaine SA (Castro-Zavala et al. 2020a), showing increased excitability in the nucleus accumbens (increased GluA1/GluA2 ratio), while MSEW-exposed females did not express changes. In the present study, we do confirm that females show resilience to this type of stress.

The mPFC is a region that modulates cognitive and executive functions, including inhibitory control (Narayanan and Laubach 2017). Recent studies show that maternal separation in mice induces depression-like behaviours as well as a reduction of serotonin and dopamine levels in the frontal cortex (Récamier-Carballo et al. 2017). Additionally, animal models of depression suggest a reduced glutamate level in the PFC of depressed mice (Belin et al. 2008), as well as reduced glutamate and glutamate/glutamine levels in depressed rats (Li et al. 2008). Clinical evidence reported increased Gria2 mRNA levels in PFC in patients with

major depression of both sexes, and no changes in Gria1 expression (Kleinman et al. 2015). Additionally, it was reported reduced glutamate/glutamine and GABA levels in the PFC of depressed patients (Hasler et al. 2007). These previous studies are in accordance with the fact that the glutamatergic system, especially in the frontal cortex, plays a key role in the modulation of the depressive phenotype.

Our present results showed a negative correlation between Gria2 in the mPFC and depression-like behaviour, meaning that a decreased Gria2 correlates with increased immobility time in the TST. Our biochemical analysis also showed a significative reduction in the Gria2 levels of mice exposed to MSEW of both sexes. Additionally, we observed a significant reduction in the Gria2 mRNA levels of MSEW-exposed male mice that evidences a significant mood alteration. However, we did not observe significant differences in GluA1 or GluA2 protein level in MSEW exposed mice. Our results are in accordance with Ganguly et al. (2019) who reported that males maternally separated showed decreased in mPFC-Gria2 expression compared to control males. Likewise, they observed that females in general, showed higher GluA2 protein level than male mice, similar to our findings.

In accordance with our results, a study using a model of depression in rats evidenced a dysregulation in glutamate neurotransmission but without changes in the levels of GluA1 or GluA2 in the mPFC of depressed rats (Treccani et al. 2016). Additionally, other authors reported no changes in Gria1 mRNA level in the mPFC of depressed mice (Belin et al. 2008) in agreement with our results.

Epidemiologic studies showed that childhood adversity increased the risk of depression by 28.4% but also to illicit drug use by 16.5% (Anda et al. 2006). Animal studies exploring how stressful situations influence addiction-like behaviours described that acute and chronic stress alters the phosphorylation of GluA2, affecting the function of the AMPA receptor but no the GluA1/GluA2 ratio in the nucleus accumbens and hippocampus (Ellis et al. 2017; Caudal et al. 2010; Caudal et al. 2016). This evidence could explain why we not able to find differences in the GluA1/GluA2 ratio. It is possible that MSEW-exposed male mice showed decreased-induced phosphorylation of GluA2, which did not modify the GluA2 levels, but altered indirectly the excitability of the AMPA receptor function, in accordance with our original hypothesis.

Caffino et al. (2015) report that a single cocaine exposure in adolescent rats can reduce the number of dendritic spines without any changes of GluA1 or GluA2 in the total mPFC homogenate. Other studies employing the cocaine SA paradigm reported that disruption of the GluA2 phosphorylation potentiated the acquisition of cocaine SA (Ellis et al. 2017). In the current work, the biochemical analysis yielded a *sex* × *rearing* interaction for the GluA1, but any alteration induced *per se* by the drug exposure (Figure 5A). However, these results indicate that MSEW females have increased GluA1 than SN females and MSEW males. The behavioural results for the cocaine SA evidenced that females, independently of the early-life stress exposure have increased impulsivity (less response efficiency).

Clinical studies evaluate the levels of glutamate in lumbar cerebrospinal fluid (CSF) in subjects diagnosed with personality disorder and healthy volunteers (Coccaro et al. 2013). They observed a direct correlation

between CSF glutamate levels and impulsivity and/or aggression, confirming the key role-playing by glutamate in the regulation of impulsivity (Coccaro et al. 2013). Moreover, it was observed that cocaine-dependent patients showed a negative correlation between the activation of the frontoparietal network (network related with the inhibition control) and the dependence severity (Barrós-Loscertales et al. 2019). In the present study, we observed that female mice have higher GluA1 protein expression at basal level (Figure 3D), which could be interpreted as greater glutamatergic activity in the mPFC than males. This fact could explain why females showed higher impulsivity than males due to increased glutamatergic activity in this brain area that seems to be related to the impulsivity regulation. Our hypothesis is in line with the association between the positive correlation between impulsivity and the number of AMPArs observed in the post-mortem anterior cingulate cortex of alcoholics (Kärkkäinen et al. 2013).

Following our suggestion, it could be expected that MSEW males exposed to cocaine, show an increase in the levels of GluA1. However, as discussed above, there is a possibility that the differences in the response efficiency during the cocaine SA between the SN males and the MSEW male mice can be explained by changes in the phosphorylation of GluA2. In the case of the MSEW females, the increased basal level of GluA1 could be compensating the hyperphosphorylation of GluA2, avoiding further increases on impulsivity for cocaine-seeking due to MSEW. Our hypothesis is in accordance with previous studies showing that methylphenidate enhances the response inhibition in rats because of the increased expression of AMPArs in the PFC (Zhang et al. 2017); that increased

AMPArs contributes to modulate the activity of the NMDA receptor in order to learn to inhibit a response (Hayton et al. 2010).

Our results are also in line with recent findings in which mutant mice lacking GRIP1 in the mPFC, a scaffolding protein that stabilizes GluA2 at the surface, showed increased GluA2-containing AMPArs in the cell membrane, and increased cocaine intake during the SA paradigm (Wickens et al. 2019). Moreover, they observed that these effects were cocaine specific and GRIP1 does not influence natural reward-seeking, as we observed in the current work.

Taken together, our results propose that female mice exhibit increased basal mPFC glutamatergic function, which potentiates impulsivity to cocaine consumption during the SA. Additionally, MSEW alters the functionality of the glutamatergic circuits in male mice, increasing the glutamatergic excitability (GluA1/GluA2) in an indirect way. We also propose that MSEW-exposed males showed higher GluA2-containing AMPArs due to the altered GluA2 phosphorylation, which avoid the internalization of this subunit. Additionally, we suggest that early-life stress affects several molecular mechanisms avoiding the ability to stabilize GluA2 in the synaptic surface. Our study evidences the underlying molecular mechanisms of the glutamatergic system regulating impulse control disorders, like cocaine use disorder and their relationship with the depression phenotype.

In addition, the present study provides novel evidence regarding the molecular mechanisms altered in the glutamatergic system in the prefrontal cortex of mice exposed to early-life stress during the first

postnatal days. These alterations could underlie the higher cocaine motivation due to modifications in the inhibition control.

Acknowledgements

This study was supported by the Ministerio de Economia y Competitividad (grant number SAF2016-75966-R-FEDER), Ministerio de Sanidad (Retic-ISCIII, RD16/017/010 and Plan Nacional sobre Drogas 2018/007) to O.V., and the Consejo Nacional de Ciencia y Tecnología in Mexico (CONACYT) (CB-2015-255317 to A.C.-M., 582196 for L.M.-M. and 276577 for A.C-Z). The Department of Experimental and Health Sciences (UPF) is an "Unidad de Excelencia María de Maeztu" funded by the MINECO (Ref. CEXS-2018-000792).

The authors thank Inés Gallego-Landin for her English proofreading and editing of the manuscript.

Author contributions

A.C-Z. and O.V. were responsible for the study concept and design. A.C-Z., A.M-S. and L.M-M. carried out the experimental studies. A.C-Z and O.V. drafted the manuscript and A.C-Z, O.V and A.C.-M. participated in the interpretation of findings. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest

The authors declare no conflicts of interest.

References

Adams T., Rapinda K. K., Frohlich J. R., O'Connor R. M., Keough M. T. (2019) Impulsivity moderates the effect of social anxiety on in-lab alcohol craving. *Addict. Behav.* **97**, 70–76.

Anda R. F., Felitti V. J., Bremner J. D., Walker J. D., Whitfield C., Perry B. D., Dube S. R., Giles W. H. (2006) The enduring effects of abuse and related adverse experiences in childhood: A convergence of evidence from neurobiology and epidemiology. *Eur. Arch. Psychiatry Clin. Neurosci.* **256**, 174–186.

Barkus C., Feyder M., Graybeal C., Wright T., Wiedholz L., Izquierdo A., Kiselycznyk C., et al. (2012) Do GluA1 knockout mice exhibit behavioral abnormalities relevant to the negative or cognitive symptoms of schizophrenia and schizoaffective disorder? *Neuropharmacology* **62**, 1263–1272.

Barrós-Loscertales A., Costumero V., Rosell-Negre P., Fuentes-Claramonte P., Llopis-Llacer J.-J., Bustamante J. C. (2019) Motivational factors modulate left frontoparietal network during cognitive control in cocaine addiction. *Addict. Biol.*, e12820.

Belin D., Mar A. C., Dalley J. W., Robbins T. W., Everitt B. J. (2008) High impulsivity predicts the switch to compulsive cocaine-taking. *Science* **320**, 1352–5.

Bian Y., Yang L., Wang Z., Wang Q., Zeng L., Xu G. (2015) Repeated three-hour maternal separation induces depression-like behavior and affects the expression of hippocampal plasticity-related proteins in C57BL/6N mice. *Neural Plast.* **2015**, 5–11.

Bowers M. S., Chen B. T., Bonci A. (2010) AMPA receptor synaptic plasticity induced by psychostimulants: the past, present, and therapeutic future. *Neuron* **67**, 11–24.

Brodsky B. S., Oquendo M., Ellis S. P., Haas G. L., Malone K. M., Mann J. J. (2001) The relationship of childhood abuse to impulsivity and suicidal behavior in adults with major depression. *Am. J. Psychiatry* **158**, 1871–1877.

Butelman E. R., Chen C. Y., Conybeare R. A., Brown K. G., Fry R. S., Kimani R., Rosa J. C. da, Ott J., Kreek M. J. (2019) Are Trait Impulsivity and Exposure to Cannabis or Alcohol Associated With the Age of Trajectory of Cocaine Use? A Gender-Specific Dimensional Analysis in Humans With Cocaine Dependence Diagnosis. *Exp. Clin. Psychopharmacol.* **28(3)**, 317–327.

Caffino L., Giannotti G., Malpighi C., Racagni G., Fumagalli F. (2015) Short-term withdrawal from developmental exposure to cocaine activates the glucocorticoid receptor and alters spine dynamics. *Eur. Neuropsychopharmacol.* **25**, 1832–1841.

Cardenas-Perez R. E., Fuentes-Mera L., la Garza A. L. de, Torre-Villalvazo I., Reyes-Castro L. A., Rodriguez-Rocha H., Garcia-Garcia A., et al. (2018) Maternal overnutrition by hypercaloric diets programs hypothalamic mitochondrial fusion and metabolic dysfunction in rat male offspring. *Nutr. Metab. (Lond).* **15**, 38.

Castro-Zavala A., Martín-Sánchez A., Luján M. Á., Valverde O. (2020a) Maternal separation increases cocaine intake through a mechanism involving plasticity in glutamate signalling. *Addict. Biol.* May 2019,

e12911.

Castro-Zavala A., Martín-Sánchez A., Valverde O. (2020b) Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice. *Eur. Neuropsychopharmacol.* **32**, 12–24.

Caudal D., Godsil B. P., Mailliet F., Bergerot D., Jay T. M. (2010) Acute Stress Induces Contrasting Changes in AMPA Receptor Subunit Phosphorylation within the Prefrontal Cortex, Amygdala and Hippocampus. *PLoS One* **5**, e15282.

Caudal D., Rame M., Jay T. M., Godsil B. P. (2016) Dynamic Regulation of AMPAR Phosphorylation In Vivo Following Acute Behavioral Stress. *Cell. Mol. Neurobiol.* **36**, 1331–1342.

Coccaro E. F., Lee R., Vezina P. (2013) Cerebrospinal fluid glutamate concentration correlates with impulsive aggression in human subjects. *J. Psychiatr. Res.* **47**, 1247–1253.

Corruble E., Benyamina A., Bayle F., Falissard B., Hardy P. (2003) Understanding impulsivity in severe depression? A psychometrical contribution. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **27**, 829–833.

Dalley J. W., Ersche K. D. (2019) Neural circuitry and mechanisms of waiting impulsivity: relevance to addiction. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **374**, 20180145.

Dalley J. W., Everitt B. J., Robbins T. W. (2011) Impulsivity, compulsivity, and top-down cognitive control. *Neuron* **69**, 680–94.

Dent C. L., Isles A. R. (2014) An overview of measuring impulsive

behavior in mice. *Curr. Protoc. Mouse Biol.* **4**, 35–45.

Ellis A. S., Fosnocht A. Q., Lucerne K. E., Briand L. A. (2017) Disruption of GluA2 phosphorylation potentiates stress responsivity. *Behav. Brain Res.* **333**, 83–89.

Fattore L., Piras G., Corda M. G., Giorgi O. (2009) The Roman High- and Low-Avoidance Rat Lines Differ in the Acquisition, Maintenance, Extinction, and Reinstatement of Intravenous Cocaine Self-Administration. *Neuropsychopharmacology* **34**, 1091–1101.

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.

Ganguly P., Honeycutt J. A., Rowe J. R., Demaestri C., Brenhouse H. C. (2019) Effects of early life stress on cocaine conditioning and AMPA receptor composition are sex-specific and driven by TNF. *Brain. Behav. Immun.* **78**, 41–51.

George E. D., Bordner K. A., Elwafi H. M., Simen A. A. (2010) Maternal separation with early weaning: a novel mouse model of early life neglect. *BMC Neurosci.* **11**, 123.

Giorgi O., Corda M. G., Fernández-Teruel A. (2019) A Genetic Model of Impulsivity, Vulnerability to Drug Abuse and Schizophrenia-Relevant Symptoms With Translational Potential: The Roman High- vs. Low-Avoidance Rats. *Front. Behav. Neurosci.* **13**, 145.

Giorgi O., Piras G., Lecca D., Corda M. G. (2005) Behavioural effects of acute and repeated cocaine treatments: A comparative study in sensitisation-prone RHA rats and their sensitisation-resistant RLA counterparts. *Psychopharmacology (Berl).* **180**, 530–538.

Goffer Y., Xu D., Eberle S. E., D'amour J., Lee M., Tukey D., Froemke R. C., Ziff E. B., Wang J. (2013) Calcium-Permeable AMPA Receptors in the Nucleus Accumbens Regulate Depression-Like Behaviors in the Chronic Neuropathic Pain State. *J. Neurosci.* **33**, 19034–19044.

Gracia-Rubio I., Martinez-Laorden E., Moscoso-Castro M., Milanés V., Laorden L., Valverde O. (2016a) Maternal Separation Impairs Cocaine-Induced Behavioural Sensitization in Adolescent Mice. *PLoS One* **11**, e0167483.

Gracia-Rubio I., Moscoso-Castro M., Pozo O. J., Marcos J., Nadal R., Valverde O. (2016b) Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **65**, 104–17.

Granö N., Keltikangas-Järvinen L., Kouvonen A., Virtanen M., Elovainio M., Vahtera J., Kivimäki M. (2007) Impulsivity as a predictor of newly diagnosed depression. *Scand. J. Psychol.* **48**, 173–9.

Haas A. L., Peters R. H. (2000) Development of substance abuse problems among drug-involved offenders: evidence for the telescoping effect. *J. Subst. Abuse* **12**, 241–253.

Hasler G., Veen J. W. Van Der, Tumonis T., Meyers N., Shen J.,

Drevets W. C. (2007) Reduced prefrontal glutamate/glutamine and γ-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. *Arch. Gen. Psychiatry* **64**, 193–200.

Hayton S. J., Lovett-Barron M., Dumont E. C., Olmstead M. C. (2010) Target-specific encoding of response inhibition: Increased contribution of AMPA to NMDA receptors at excitatory synapses in the prefrontal cortex. *J. Neurosci.* **30**, 11493–11500.

Hynes T. J., Thomas C. S., Zumbusch A. S., Samson A., Petriman I., Mrdja U., Orr A., et al. (2018) Early life adversity potentiates expression of addiction-related traits. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **87**, 56–67.

James S. L., Abate D., Abate K. H., Abay S. M., Abbafati C., Abbasi N., Abbastabar H., et al. (2018) Global, regional, and national incidence, prevalence, and years lived with disability for 354 Diseases and Injuries for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **392**, 1789–1858.

Jupp B., Jones J. A., Dalley J. W. (2020) Modelling Differential Vulnerability to Substance Use Disorder in Rodents: Neurobiological Mechanisms. *Handb. Exp. Pharmacol.* **258**, 203–230.

Kärkkäinen O., Kupila J., Häkkinen M., Laukkanen V., Tupala E., Kautiainen H., Tiihonen J., Storvik M. (2013) AMPA receptors in post-mortem brains of Cloninger type 1 and 2 alcoholics: A whole-

hemisphere autoradiography study. *Psychiatry Res. - Neuroimaging* **214**, 429–434.

Kauer J. A., Malenka R. C. (2007) Synaptic plasticity and addiction. *Nat. Rev. Neurosci.* **8**, 844–858.

Kleinman J. E., Sodhi M. S., Hyde T. M., Deep-Soboslay A., Gray A. L. (2015) Sex differences in glutamate receptor gene expression in major depression and suicide. *Mol. Psychiatry* **20**, 1057–1068.

Koob G. F., Zorrilla E. P. (2010) Neurobiological mechanisms of addiction: focus on corticotropin-releasing factor. *Curr. Opin. Investig. Drugs* **11**, 63–71.

Li C. X., Wang Y., Gao H., Pan W. J., Xiang Y., Huang M., Lei H. (2008) Cerebral metabolic changes in a depression-like rat model of chronic forced swimming studied by ex vivo high resolution 1H magnetic resonance spectroscopy. *Neurochem. Res.* **33**, 2342–2349.

Liu C., Hao S., Zhu M., Wang Y., Zhang T., Yang Z. (2018) Maternal Separation Induces Different Autophagic Responses in the Hippocampus and Prefrontal Cortex of Adult Rats. *Neuroscience* **374**, 287–294.

Martínez-Rivera A., Hao J., Tropea T. F., Giordano T. P., Kosovsky M., Rice R. C., Lee A., et al. (2017) Enhancing VTA Ca v 1.3 L-type Ca2+channel activity promotes cocaine and mood-related behaviors via overlapping AMPA receptor mechanisms in the nucleus accumbens. *Mol. Psychiatry* **22**, 1735–1745.

Nakamura K., Kurasawa M., Shirane M. (2000) Impulsivity and AMPA

receptors: aniracetam ameliorates impulsive behavior induced by a blockade of AMPA receptors in rats. *Brain Res.* **862**, 266–9.

Narayanan N. S., Laubach M. (2017) Inhibitory Control: Mapping Medial Frontal Cortex. *Curr. Biol.* **27**, R148–R150.

Nicholls J., Staiger P. K., Williams J. S., Richardson B., Kambouropoulos N. (2014) When social anxiety co-occurs with substance use: Does an impulsive social anxiety subtype explain this unexpected relationship? *Psychiatry Res.* **220**, 909–914.

Perry J. L., Larson E. B., German J. P., Madden G. J., Carroll M. E. (2005) Impulsivity (delay discounting) as a predictor of acquisition of IV cocaine self-administration in female rats. *Psychopharmacology (Berl).* **178**, 193–201.

Portero-Tresserra M., Gracia-Rubio I., Cantacorps L., Pozo O. J., Gómez-Gómez A., Pastor A., López-Arnau R., la Torre R. de, Valverde O. (2018) Maternal separation increases alcohol-drinking behaviour and reduces endocannabinoid levels in the mouse striatum and prefrontal cortex. *Eur. Neuropsychopharmacol.* **28**, 499–512.

Récamier-Carballo S., Estrada-Camarena E., López-Rubalcava C. (2017) Maternal separation induces long-term effects on monoamines and brain-derived neurotrophic factor levels on the frontal cortex, amygdala, and hippocampus: differential effects after a stress challenge. *Behav. Pharmacol.* **28**, 545–557.

Río-Álamos C., Piludu M. A., Gerbolés C., Barroso D., Oliveras I., Sánchez-González A., Cañete T., et al. (2019) Volumetric brain

differences between the Roman rat strains: Neonatal handling effects, sensorimotor gating and working memory. *Behav. Brain Res.* **361**, 74–85.

Rømer Thomsen K., Callesen M. B., Hesse M., Kvamme T. L., Pedersen M. M., Pedersen M. U., Voon V. (2018) Impulsivity traits and addiction-related behaviors in youth. *J. Behav. Addict.* **7**, 317–330.

Treccani G., Gaarn du Jardin K., Wegener G., Müller H. K. (2016) Differential expression of postsynaptic NMDA and AMPA receptor subunits in the hippocampus and prefrontal cortex of the flinders sensitive line rat model of depression. *Synapse* **70**, 471–474.

UNODC (2019) *World Drug Report 2019*.

Vannan A., Powell G. L., Scott S. N., Pagni B. A., Neisewander J. L. (2018) Animal Models of the Impact of Social Stress on Cocaine Use Disorders. *Int. Rev. Neurobiol.* **140**, 131–169.

Vetulani J. (2013) Early maternal separation: a rodent model of depression and a prevailing human condition. *Pharmacol. Rep.* **65**, 1451–61.

WHO (2017) *Depression and Other Common Mental Disorders: Global Health Estimates*.

Wickens M. M., Deutschmann A. U., McGrath A. G., Parikh V., Briand L. A. (2019) Glutamate receptor interacting protein acts within the prefrontal cortex to blunt cocaine seeking. *Neuropharmacology* **157**, 107672.

Zhang D.-D., Zhang Y.-Q., Zhang X.-H. (2017) Prefrontal AMPA receptors are involved in the effect of methylphenidate on response inhibition in rats. *Nat. Publ. Gr.* **39**, 607–615.

FIGURE LEGENDS

Figure 1. Schematic representation of the experimental schedule. **(**A) Schematic representation of the MSEW model and the (B) timeline in which the experiments were performed.

Figure 2. Effects of MSEW on the TST. Total immobility time in SN and MSEW adult male and female mice. *Sex* main effect of the ANOVA (★★p<0.01). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines (##p<0.01, ###p<0.001). Data are expressed as mean ± SEM (n=10-12 per group).

Figure 3. mRNA and protein levels of two AMPArs subunits in the mPFC of SN and MSEW drug-naïve mice. (A) Heatmap representing mRNA expression of Gria1, Gria2 and Gria1/Gria2 ratio in the mPFC of SN and MSEW mice relative to SN males as determined by qPCR. (B) Scatter blot illustrating the negative correlation between Gria2 and depression-like behaviour in mice. (C) Linear regression representing the relationship between depression-like behaviour and Gria2 expression in mice (Y= -0.0022X + 1.075). Mean fold change relative to SN males of (D) GluA1, (E) GluA2 and (F) GluA1/GluA2 protein levels in the mPFC of drug-naïve mice. *Sex* main effect of the ANOVA (★p<0.05). For the qPCR (n=10 per group, run in duplicate) and for the western blot (n=5 per group, run in triplicate). Data are expressed as mean ± SEM.

Figure 4. Percentage of response efficiency in the SA paradigm of SN and MSEW mice. Percentage of response efficiency as an indirect measure of impulsivity in the (A) cocaine SA (n=20-30 per group) or

the (B) food SA procedure (n=10-18 per group). *Sex* main effect of the ANOVA (★★p<0.01). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines (##p<0.01). High response efficiency scores mean less impulsivity whereas low response efficiency signifies more impulsivity. Data are expressed as mean ± SEM.

Figure 5. Fold change of GluA1, GluA2 and GluA1/GluA2 ratio in the mPFC of SN and MSEW mice. Mean fold change relative to SN males drug-naïve of (A) GluA1, (B) GluA2 and (C) GluA1/GluA2 levels in the mPFC. Representative western blot showing protein levels of (D) GluA1 and (E) GluA2 in the mPFC. The protein of interest in red and tubulin in green. *Sex* main effect of the ANOVA (★p<0.05, ★★★p<0.001). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* (#p<0.05, ###p<0.001). Data are expressed as mean ± SEM (n=5, run in triplicate).

Figure 1.

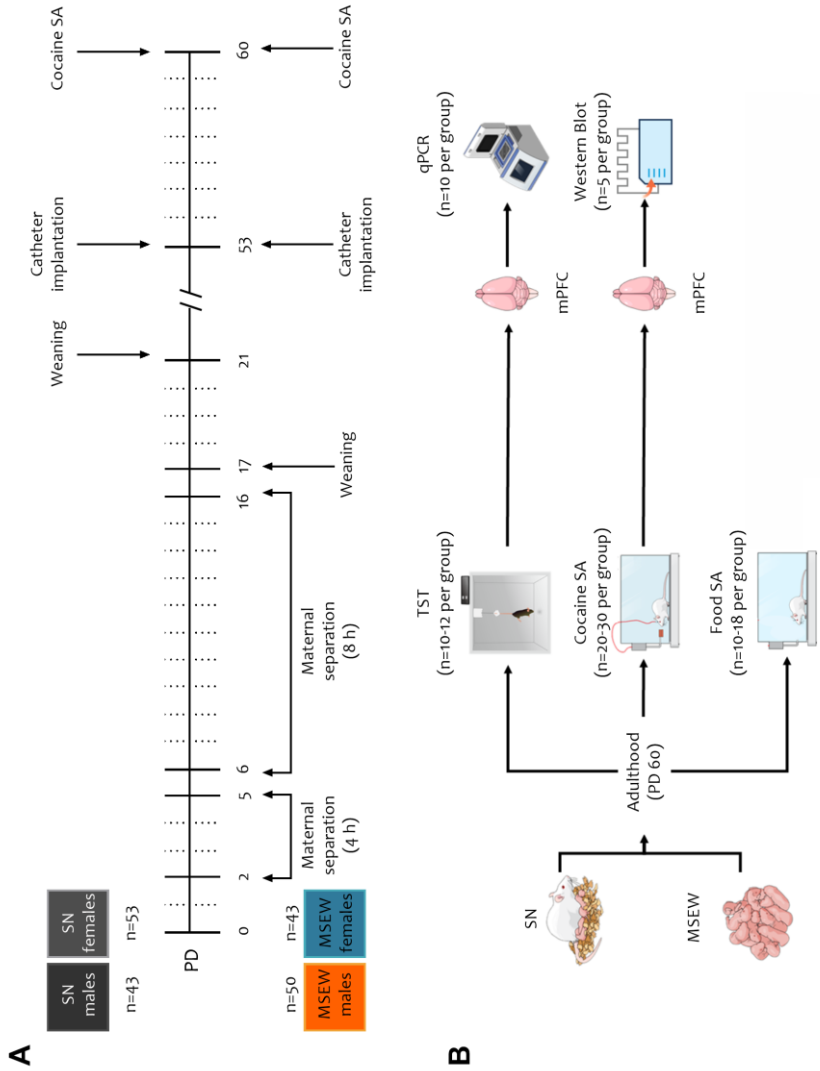

A
SN males
SN females
n=43
n=53
PD
0
2
5
6
16
17
21
53
60
Weaning
Catheter implantation
Cocaine SA
Maternal separation (4 h)
Maternal separation (8 h)
n=50
n=43
MSEW males
MSEW females
B
SN
MSEW
Adulthood (PD 60)
TST (n=10-12 per group)
Cocaine SA (n=20-30 per group)
Food SA (n=10-18 per group)
qPCR (n=10 per group)
Western Blot (n=5 per group)
mPFC

Figure 2.

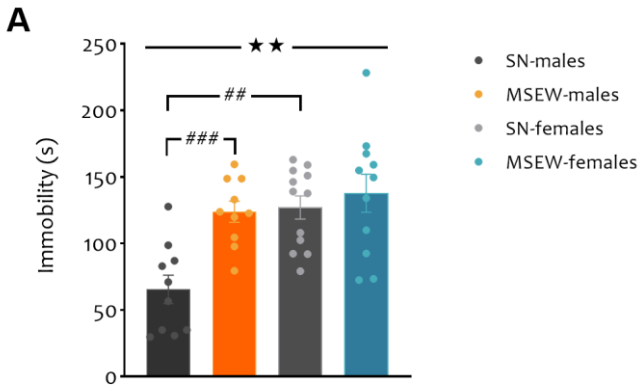

A
Immobility (s)
250
200
150
100
50
0
★★
##
###
SN-males
MSEW-males
SN-females
MSEW-females

Figure 3.

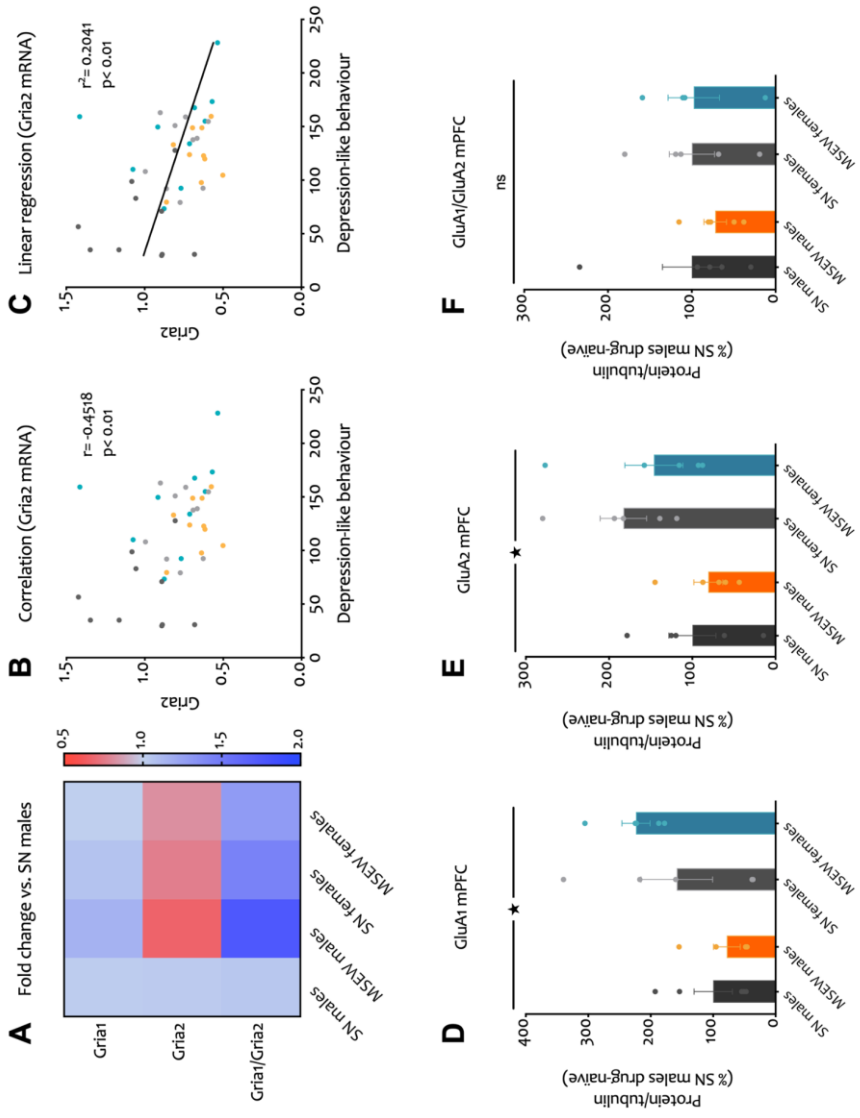

A
Fold change vs. SN males
Gria1
Gria2
Gria1/Gria2
SN males
MSEW males
SN females
MSEW females
B
Correlation (Gria2 mRNA)
r= -0.4518
p< 0.01
Gria2
Depression-like behaviour
C
Linear regression (Gria2 mRNA)
r²= 0.2041
p< 0.01
Gria2
Depression-like behaviour
D
GluA1 mPFC
Protein/tubulin
(% SN males drug-naïve)
E
GluA2 mPFC
Protein/tubulin
(% SN males drug-naïve)
F
GluA1/GluA2 mPFC
ns
Protein/tubulin
(% SN males drug-naïve)

Figure 4.

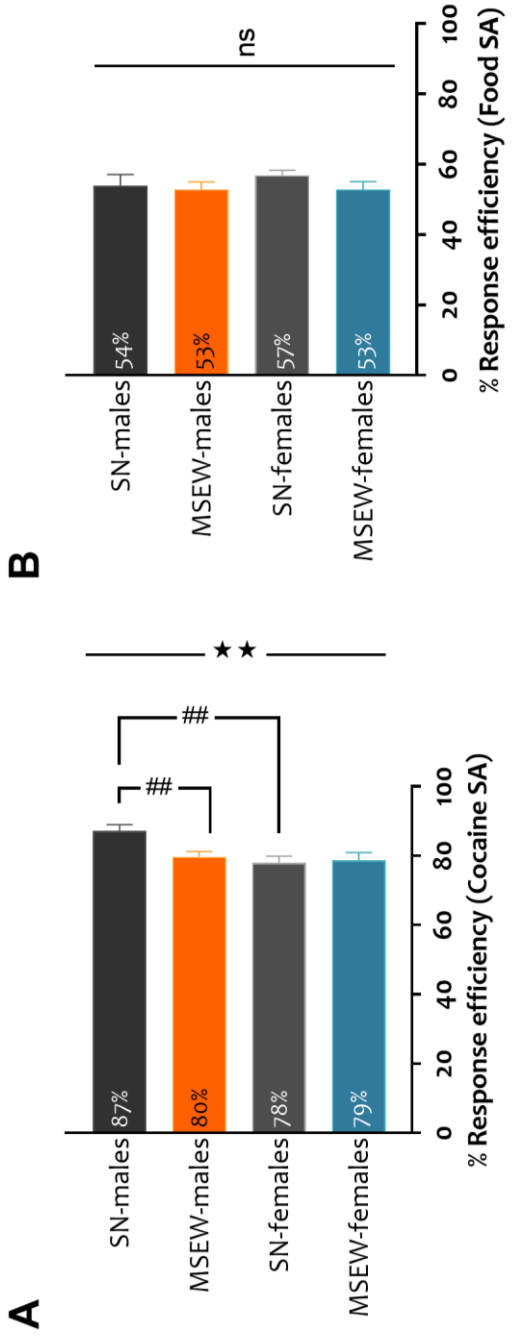

B
ns
SN-males 54%
MSEW-males 53%
SN-females 57%
MSEW-females 53%
0 20 40 60 80 100
% Response efficiency (Food SA)
A
★★
##
##
SN-males 87%
MSEW-males 80%
SN-females 78%
MSEW-females 79%
0 20 40 60 80 100
% Response efficiency (Cocaine SA)

Figure 5.

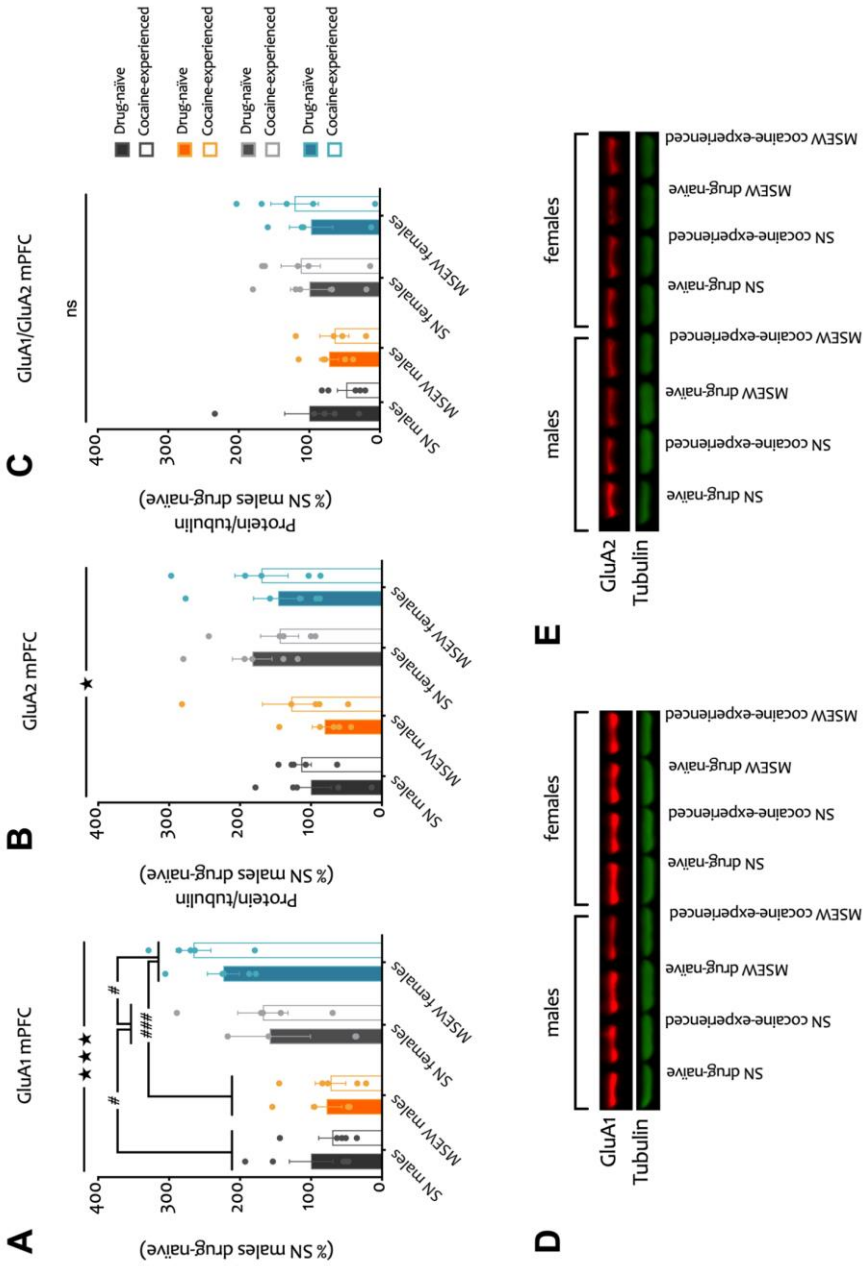

A
GluA1 mPFC
Protein/tubulin
(% SN males drug-naïve)
B
GluA2 mPFC
C
GluA1/GluA2 mPFC
ns
Drug-naïve
Cocaine-experienced
SN males
MSEW males
SN females
MSEW females
D
GluA1
Tubulin
E
GluA2
Tubulin
males
females
SN drug-naïve
SN cocaine-experienced
MSEW drug-naïve
MSEW cocaine-experienced

Table 1. Antibodies

Antibody	# Catalogue	RRIDs	Dilution	Company
GluA2	AB1768	AB_2313802	1:1000	Milipore
GluA1	ABN241	AB_2721164	1:1000	Milipore
β-tubulin	556321	AB_396360	1:5000	BD Biosciences
goat anti-mouse IgG H&L (IRDye 800)	ab216772	AB_2857338	1:2500	Abcam
goat anti-rabbit IgG H&L (DyLight 680)	611-144-002	AB_1660962	1:2500	Rockland

Table 2. Primers for qPCR

	Primer sequence (5'→3')
Gria1 Forward	ACA ACT CAA GCG TCC AGA ATA G
Gria1 Reverse	CAT AGC GGT CAT TGC CTT CA
Gria2 Forward	CCT TTC TTG ATC CTT TAG CCT ATG A
Gria 2 Reverse	CTG CTG ACC AGG AAT AAA ACT ACA CT
36B4 Forward	TCC AGG CTT TGG GCA TCA
36B4 Reverse	CTT TAT CAG CTG CAC ATC ACT CAG A

DISCUSSION

Early-life adversity has long-lasting effects that persist until adulthood, affecting the brain function, cognitive and emotional development (Krugers et al., 2017). Moreover, child abuse and neglect are risk factors to develop emotional disorders such as depression or SUD (Nelson and Gabard-Durnam, 2020; Tarantola, 2018).

Epidemiological studies show that people diagnosed with depression are more prone to use drugs to ameliorate their symptoms (Torrens et al., 2015). However, SUD also trigger and potentiate the risk of developing depression (Torrens et al., 2015). This shows the impact that understanding of such comorbidity has on the evolution of both diseases. In humans, experienced maltreatment during childhood increase 54% the probability to develop depression and 64% to suffer addiction to illicit drugs (Dube et al., 2003a). In this regard, cocaine is the most consumed psychostimulant worldwide and the chance to become an addict increases two fold in individuals with a history of childhood maltreatment (Ducci et al., 2009). Moreover, they have a 90% of probability to relapse in CUD after treatment compared with the 45% in people without childhood maltreatment (Hyman et al., 2008).

Growing evidence yield that early-life adversity-induced synaptic plasticity in the mesocorticolimbic pathway that contributes to these pathological behaviours (Eagle et al., 2019; Salamone and Correa, 2012). The components of this pathway (mPFC, NAc and VTA, among others) are closely related in goal-directed actions, integrating excitatory (glutamatergic) and neuromodulator input along with local inhibitory control to reward-related processes (Turner et al., 2018).

Recently, disruptions in AMPA receptors composition in the brain reward system have been associated with motivational symptoms of depression and substance abuse, being proposed as a common target linking addiction and mood disorders (Goffer et al., 2013; Martínez-Rivera et al., 2017).

Animal models are a useful tool to understand maladaptation underlying pathological behaviours. In rodents, MSEW is a reliable animal model of early-life stress (George et al., 2010) and depression-like behaviour (Vetulani, 2013), allowing to explore the consequences that these phenomena have in addictive behaviours (Gracia-Rubio, 2016; Gracia-Rubio et al., 2016b, 2016a; Portero-Tresserra et al., 2018).

Although the evident impact that early-life stress has in the developing of addictive behaviour, the molecular mechanisms are still not fully understood. Therefore, it is necessary to explore the neurobiological effects to find a possible clinical target to avoid the deleterious consequences of this kind of stress and develop a more effective therapeutic interventions for this important comorbidity (depression and addiction).

Hence, this thesis pursued *to evaluate the long-term effects of MSEW in factors related to neuroplasticity, depression and cocaine addiction behaviour, as well as their underlying molecular mechanisms*.

1. Behavioural consequences of MSEW in male mice during the acquisition, extinction and reinstatement phases of the cocaine SA paradigm.

To understand the consequences of early-life stress experiences in cocaine addictive behaviour, we decided to perform the MSEW procedure in CD1 mice. MSEW reproduce in animals, the effects of childhood maltreatment or neglect experienced by humans. In addition, we select the SA procedure as strategy for voluntary cocaine consumption in a dose of 1.0 mg/kg mice.

In humans, approximately 20% of cocaine users become addicts (Wagner and Anthony, 2002), but the likelihood of shifting from cocaine use to CUD increases two folds in individuals with a history of childhood maltreatment (Ducci et al., 2009). Previous studies showed that maternal separation enhances the acquisition of cocaine SA (Moffett et al., 2006), increases cocaine intake (Lynch et al., 2005) and elevates the number of responses during the reinstatement phase (Lynch et al., 2005). Here, we find that MSEW increases the reinforcing effects of cocaine in the SA paradigm including cocaine intake, number of nosepokes and infusions performed during the acquisition phase. Moreover, we observed that MSEW increased the percentage of addicted animals in the cocaine SA, in a similar way observed in the human population with history of childhood maltreatment. While the percentage of SN mice acquiring cocaine SA was 32%, the rate increased to 51% in MSEW mice the percentage of the acquisition.

Clinical research reveal that childhood maltreatment negatively impacts cocaine relapse even after long periods of abstinence (Hyman et al.,

2008). Our results show that MSEW decreased the capacity to extinguish the cocaine-seeking behaviour. In the SN group, the 100% of the animals extinguished cocaine SA behaviour, while only 65% of the MSEW mice achieved the extinction criteria. Moreover, along all the days of the extinction phase, MSEW mice showed a higher number of responses, evidencing an increased cocaine-seeking than the SN group.

Regard to the relapse rate, epidemiological studies report cocaine relapse rate around 45% in general population (Back et al., 2010). However, for individuals who have suffered childhood maltreatment the figure rises to around 90% (Hyman et al., 2008). Although our results show a similar reinstatement percentage for both groups of mice (92% in MSEW vs. 86% in SN), cocaine-seeking behaviour during reinstatement was higher in the MSEW group.

Previously, Romano-López et al. (2015) showed that maternal separation induced several changes in the PFC and in the NAc of rats, suggesting a deregulation of the dopaminergic system that facilitates addictive behaviours. They reported that maternal separation rises the NAc dopamine D2R expression, TH levels and the dendritic length (Romano-López et al., 2015).

In our laboratory, we also displayed alterations in the mesolimbic dopamine system in the MSEW model (Gracia-Rubio et al., 2016a). Gracia-Rubio et al. (2016a) reported decreased expression of D2R in the NAc and higher expression of Nurr1 in the VTA, a transcriptional factor that activates the dopamine synthesis and the transcription of DAT protein, vesicular monoamine transporter and TH (Bissonette and

Roesch, 2016). Moreover, the results yield that following cocaine-induced CPP, the levels of Nurr1 in the VTA decreased significatively (Gracia-Rubio et al., 2016a), in accordance with clinical studies reporting decreased Nurr1 expression in midbrain dopaminergic neurons of cocaine abusers (Bannon et al., 2002).

Moreover, Larsen et al. (2016) reported that Nurr1 gene shows a CpG islands with higher GC content in the promoter region meaning that DNA methylation regulates the transcription of this gen (Larsen et al., 2016). In line with this, Anier et al. (2014) observed maternal separation-induced hypermethylation of genes related with cocaine-induced neuroplasticity in the NAc of adult rats, being in accordance with our recent and previous results.

Therefore, our results support that changes induced by maternal separation in the mesolimbic dopamine system contribute to generate detrimental neuroadaptations in the brain that increases cocaine addictive behaviour.

2. Changes in AMPA composition and CREB activity in the NAc and VTA of MSEW male mice, after the acquisition or reinstatement of cocaine SA.

Using the same MSEW paradigm, Gracia-Rubio et al. (2016b) reported anhedonia, higher anxiety- and depressive-like behaviour in mice. In line with such data, Goffer et al. (2013) reported an increased GluA1 expression in the NAc of depressive rats, pointing to GluA2-lacking AMPA receptors as regulators of depressive-like behaviours (Goffer et al., 2013). Moreover, a higher depression vulnerability was associated with increased GluA1/GluA2 ratio (increased AMPAr function) and

decreased GluA2 levels in the NAc of socially-stressed mice (Vialou et al., 2010). Fittingly to these observations, we observed that MSEW mice had reduced GluA2 expression but increased GluA1/GluA2 ratio and GluA1 in the NAc.

Concerning to cocaine exposition, studies in rats show increased GluA1 levels and higher GluA1/GluA2 ratio in the NAc after repeated cocaine exposure (Boudreau and Wolf, 2005). However, we did not find significative differences when we compared the GluA1, GluA2, GluA1/GluA2 basal levels with the ones measured after acquisition or reinstatement in the NAc.

On other hand, Conrad et al. (2008) demonstrated that GluA2-lacking AMPA receptors are associated with augmented excitability of NAc neurons, increased cocaine craving and a higher reinstatement of previously extinguished cocaine-seeking behaviour. Likewise, Ping et al. (2008) reported a positive association between cocaine-reinstatement rate and GluA1 expression in the NAc of rats.

In accordance with previous reports, we observed that MSEW mice, the group of animals with higher GluA1 expression, clearly stated a higher cocaine-seeking behaviour during all the phases (acquisition, extinction and reinstatement).

As exposed at the beginning of this thesis, CREB is a transcriptional factor involved in the modulation of the rewarding effects of cocaine (Carlezon et al., 1998) and the etiology of depression (Nestler et al., 2002). CREB activity induces the expression of BDNF and dynorphin, two genes that contribute to depressive-related behaviours (Nestler,

2015). In fact, dynorphin activates KORs in VTA neurons, inhibiting dopaminergic activity and contributing to anhedonia and the development of depressive symptoms (Nestler, 2015). To this effect, Gustafsson et al. (2008) showed that maternal separation in rats induces an increased expression of dynorphin in the NAc. Additionally, the gene disruption of prodynorphin or the KOR induce a potentiation of the rewarding effects of cocaine in mice (McLaughlin et al., 2003).

Being in accordance with the evidence, our data display increased pCREB expression in the NAc of MSEW mice compared with the SN group. This higher CREB activity participates in the pro-depressive phenotype of this animals. We did not find cocaine-induced changes in CREB or pCREB expression in the MSEW mice.

Corresponding to the SN group, we observed that following the acquisition of the cocaine SA behaviour, the expression of CREB in the NAc was reduced. Larson et al. (2011) reported that reduced CREB expression in NAc, accelerated the extinction of cocaine-seeking in the SA paradigm, whilst augmented CREB enhances cocaine reinforcement. This help to explain why the percentage of extinction in the SN mice was higher than the obtained by the MSEW animals.

Evaluating the alterations in the VTA, our results show that MSEW mice express lower basal GluA2 levels and higher GluA1/GluA2 basal ratio in comparison with the SN group, suggesting enhanced excitability of VTA dopaminergic neurons MSEW-induced. This is supported by previous studies in rodents which showed that enhanced excitability of VTA dopaminergic neurons is associated with a higher vulnerability to cocaine SA, depressive-like behaviour and cocaine addiction (Martínez-

Rivera et al., 2017), similar to the phenotype observed in our MSEW mice.

Regarding cocaine-induced changes, previous studies in rats have reported no modifications (Tang et al., 2004) or increased expression of GluA1 in the VTA following cocaine consumption (Choi et al., 2011). In our case, we observed a significant GluA1 decrease after the acquisition of cocaine SA in this brain region. This alteration could represent a compensatory effect to attenuate dopamine release induced by the drug. Additionally, we also observed increased levels of GluA1 after cocaine reinstatement in comparison to the GluA1 after acquisition phase.

Finally, the acquisition of cocaine SA reduced the levels of CREB and pCREB in the VTA of mice, whereas cocaine-primed reinstatement induced an increase of CREB and pCREB levels in the same brain structure. Such results are in accordance with previous studies showing that cocaine priming increased pCREB and induced reinstatement in cocaine-conditioned animals (Kreibich and Blendy, 2004). Moreover, Kreibich et al. (2009) showed that chronic stress exposure enhances cocaine rewarding effects (Kreibich et al., 2009), a process that seems to be mediated by CREB (Kreibich and Blendy, 2004). Accordingly, our results show that early-life stress induced by MSEW, reduce CREB levels and increase the pCREB/CREB ratio in the VTA, which go hand in hand with higher cocaine intake during SA acquisition.

Based on our results, we propose that MSEW enhances NAc-glutamatergic function due to the reduction in GluA2, increased GluA1 expression and a higher GluA1/GluA2 ratio. Moreover, this enhanced

glutamatergic activity will elevate pCREB levels in the NAc of MSEW mice, as well as dynorphin levels in the NAc. Such facts could potentiate the inhibitory function of GABAergic neurotransmission from the NAc to the VTA, inhibiting the release of dopamine from the VTA neurons. This could explain the fact that MSEW mice increased the cocaine intake during the acquisition phase, to could achieve greater drug-reinforcement effects.

As a result of the enhanced GABAergic function (augmented inhibition), MSEW mice will show depressive-like behaviour, higher acquisition rates and tolerance to the rewarding effects of cocaine, being more susceptibility to cocaine-seeking. However, animals reared in standard conditions, generated a protective mechanism to counteract the deleterious effects of the drug. This homeostatic mechanism was the reduction of CREB in the NAc, in order to facilitates the extinction of cocaine SA behaviour. Nevertheless, this compensatory mechanism was not developed in the MSEW mice.

Due to the changes observed in the VTA of MSEW mice (reduced GluA2 and increased GluA1/GluA2 ratio), we suggest that MSEW mice display an enhanced excitability of VTA dopaminergic neurons, probably to compensate for the higher GABAergic inhibition from the NAc. This enhanced excitability contributes to the depressive-like phenotype of MSEW mice and their higher vulnerability to cocaine SA.

Concerning cocaine, it is well known that this psychostimulant induces LTP in excitatory synapses in the VTA; such adaptations have a pivotal role in the incubation of cocaine craving and cocaine-seeking (Lüscher, 2013a). Likewise, studies in rodents have shown that cocaine abstinence

produces a stronger activation of dopamine pathways due to hyperactivity in dopamine neurotransmission.

Based on this information, we propose that during the extinction phase, GluA1 levels promote the incubation of cocaine craving, enhancing the plasticity in this brain area. Because of this AMPA subunit elevation during the abstinence, the activation of dopamine neurons in the VTA will be facilitated, resulting in a stronger cocaine-seeking during the reinstatement phase.

In accordance with our hypothesis, previous studies showed that CNQX administrated locally in discrete brain areas or systemically, are able to reduce cocaine intake and cocaine seeking behaviour. Infusions of CNQX into the core of the NAc decreased levels pressing for cocaine in rats (Suto et al., 2009b) and blocked the reinstatement of cocaine seeking induced by intra-PFC cocaine infusion in rats (Park et al., 2002). Additionally, Mahler et al. (2013) showed that microinjections of CNQX/AP-5 into the VTA of rats reduced cocaine seeking elicited by cues in rats (Mahler et al., 2013). Other studies have also reported that CNQX systemically administered (ip) modulated cocaine-induced SA (Bäckström and Hyytiä, 2003) or reinstatement to cocaine seeking (Bäckström and Hyytiä, 2006). These data clearly state the participation of the glutamatergic neurotransmission through AMPA receptors in different processes related to cocaine-induced motivation and seeking behaviour.

3. MSEW-induced sex differences in the acquisition of cocaine SA behaviour.

Clinical and preclinical research have identified sex differences in substance use and addiction-related behaviours (Giacometti and Barker, 2020). Although SUD are more common in men, women progress more rapidly from recreational use to abuse due to the telescoping effect (Haas and Peters, 2000; UNODC, 2019).

Additionally, when taking the same drug dose, the blood concentrations of drugs are different in men and women, which may contribute to the vulnerability to drug misuse and neurobehavioral consequences of drug intake (Giacometti and Barker, 2020).

Besides, as comment in chapter 3, there is an important comorbidity between depression and SUD, and both disorders interact to make each other worse (Torrens et al., 2015). Statistics show that depression and anxiety trend to be more common among women than among men (UNODC, 2018). Even though, the preclinical studies evaluating depression-like behaviour was performed predominantly in male mice (82.6%) rather than female mice (17.4%).

Moreover, evidence show that childhood adversity has different impact in women and men (Goodwill et al., 2019). Therefore, it is necessary to explore the impact of early-life adversity during the early postnatal period and the sex-driven determinants in vulnerability or resilience to depression or addiction.

For this reason, we explore MSEW-induced differences in the acquisition of cocaine SA in male and female CD1 mice.

In our study, the reinforcing effects of cocaine were more powerful in the female mice compared to their male counterparts in the SA paradigm. We observed that females showed a faster and higher percentage of SA behaviour acquisition, in addition to greater cocaine-seeking and intake, thus displaying a different progression and higher vulnerability to cocaine addiction. These results are in line with clinical data reporting that women were more susceptible to cocaine use than men (Lynch et al., 2002). Also, preclinical studies demonstrated that females have higher reinforcing effects of cocaine in the SA (Caine et al., 2004; Cummings et al., 2011; Davis et al., 2008; Johnson et al., 2019; Lynch, 2008; Peterson et al., 2014).

It is known that childhood adversity is a risk factor to develop drug addiction, but the way in which affects women and men is different (Haas and Peters, 2000; UNODC, 2018). Animal studies evaluating the effects of neonatal isolation in rats reported higher cocaine consumption in females, independently of the isolation procedure (Kosten et al., 2007, 2004; Lynch et al., 2005). Here, we also observed that females, independently of the maternal separation procedure, obtained more cocaine than males. In fact, SN females consumed a greater quantity of cocaine than SN males.

Previous data reported that isolated males rodents consume more cocaine than the control males (Kosten et al., 2007, 2004; Lynch et al., 2005). In line with this, Newman et al. (2018) reported that social isolation stress potentiates motivation and leads to increased infusions during cocaine SA only in male mice.

A recent study showed that continuous social defeat stress increased or attenuated cocaine SA in male mice, depending on the social stress response (high and low responders) (Arena et al., 2019). Therefore, the individual response to stress experience contributes to developing different reward-seeking phenotype (Arena et al., 2019). Besides, authors suggest that this could be led by changes in neural mechanisms that will induce vulnerability or resilience to the stress experience (Arena et al., 2019).

In agreement with these observations, our results yield that MSEW males performed a higher number of infusions, consumed more cocaine and acquire the cocaine SA behaviour earlier than the SN males. Nevertheless, females were not affected by the MSEW in the number of infusions, percentage of acquisition, total cocaine intake or the day of acquisition. In fact, MSEW reduced the number of infusions in females. Therefore, our study revealed that MSEW enhances cocaine-seeking behaviour and cocaine intake, albeit solely in male mice.

As conclusion, male mice were negatively affected by the MSEW, while females appeared to be more resilient to this kind of early-life stress, as previously suggested by Kikusui et al. (2005).

4. Sex-dimorphic molecular changes induced by chronic cocaine exposure and/or early-life stress in the NAc and VTA.

Albeit the existence of the telescoping effect, much of the work evaluating drug-seeking behaviours and cocaine-induced molecular changes, have been conducted in men and male rodents (Giacometti and Barker, 2020).

For this reason, we evaluated sex-differences in AMPA receptor subunit composition as well as sex-differences in cocaine-induced neuroplastic changes.

An increasing number of studies have reported that cocaine exposure induces LTP in the VTA and NAc (Hemby et al., 2005; Nestler, 2001). LTP induction triggers the exocytosis of perisynaptic AMPA receptors in the membrane in order to induce a transient activation of the NMDA receptors (Andrásfalvy et al., 2004). These new AMPARs contain GluA1 subunits (GluA2-lacking AMPARs) (Yang et al., 2010, 2008) are responsible for the full expression of the LTP (Yang et al., 2008). Once inserted, the subunit GluA1 is rapidly changed by GluA2 to stabilize the new synapse (Adesnik and Nicoll, 2007; Yang et al., 2008). Evidence show cocaine-induced translocation of GluA2 for GluA1 in the NAc; this cocaine-induced GluA1 increase contributes to seeking behaviour (Conrad et al., 2008; Kalivas, 2009; Pierce and Wolf, 2013). In accordance with such results, we observed that animals with increased GluA1 basal expression in the NAc (females) showed increased cocaine seeking.

Our results also show that MSEW males, but no females, registered a positive GluA1 fold change and higher GluA1/GluA2 ratio in NAc than the SN males, but also than MSEW females. Such molecular alterations may explain why only males exposed to this early-life stress registered increase number of infusions and cocaine intake during SA.

In line with our results, Fosnocht et al. (2019) observed that social isolation stress during adolescence, increases responding for cocaine in male mice but not in females, but also that cocaine exposure after

adolescent stress increases c-Fos expression in the NAc independently of the sex. Therefore, they suggest that this increased behavioural responsivity to cocaine could be regulated by the glutamatergic system (Fosnocht et al., 2019).

One neuroadaptation reported to compensate cocaine-induced dopamine release is the activation of CREB in the GABAergic neurons of the NAc, which induce dynorphin expression (Muschamp and Carlezon, 2013). CREB-mediated dynorphin augmentation reduces dopamine release within the NAc, acting on KOR in the VTA (Muschamp and Carlezon, 2013). Our results show that males after cocaine SA, expressed higher CREB activation (pCREB/CREB) in the NAc than females.

Like cocaine, chronic stress also activates the CREB pathway promoting depressive like-behaviour (Covington et al., 2011). Sustained elevations of CREB activity in the NAc produces anhedonia-like profile (Barrot et al., 2002). Even our results showed no significant effect of the MSEW, we observed that maternally separated mice tended to show increased CREB activation (pCREB/CREB) in the NAc, in accordance with our earlier observation that MSEW induces depressive-like behaviour (Gracia-Rubio et al., 2016b).

In addition to the alterations in the NAc, we evaluated changes in the VTA, which play a key role in the reinforcing effects of cocaine (Nestler, 2001). Cocaine-evoked VTA synaptic plasticity is mediated by changes in glutamatergic synapses, and the new protein synthesis is triggered by cocaine-induced LTP (Heshmati, 2009). Some studies have reported that cocaine SA increases GluA1 and GluA2 in rats and that GluA1

overexpression in the VTA enhances level-press behaviour in the same paradigm (Choi et al., 2011). Our results yield higher GluA1 in females after acquisition than acquired males. Additionally, we observed that MSEW increase the GluA1/GluA2 ratio in the VTA of males, but not in females. Furthermore, MSEW drug-naïve males show lower GluA2 than the SN drug-naïve males and even lower than the MSEW drug-naïve females.

Our results revealed that cocaine SA acquisition induced a negative fold change of GluA1 and GluA1/GluA2 ratio in VTA of males, whereas the cocaine exposure did not modify GluA1 or GluA1/GluA2 ratio in females. In this way, GluA1 transcription is regulated by CREB (Olson et al., 2005), and CREB activation modulates the rewarding effects of cocaine in the VTA (Tang et al., 2003). Moreover, TH, the rate-limiting enzyme in dopamine synthesis, is another target gene for CREB in the VTA (Nestler, 2001; Olson et al., 2005). Accordingly, our results show that drug-experienced males registered lower pCREB than drug-naïve males, and, consequently, GluA1 expression. The same relation (pCREB→GluA1) was present for females- and males- drug-experienced. Results yield that levels of pCREB and GluA1 were increasing in females after acquisition in comparison with the levels of males drug-experienced.

Based in our results, we propose that the increased expression of NAc GluA1 basal level in female mice, allow them to generate a faster and stronger cocaine-induced LTP, thus explaining why females acquire cocaine SA behaviour earlier and in higher percentage than males. Moreover, the MSEW-induced male-specific increase of GluA1, yield

the idea that GluA1 is a key marker indicating a higher vulnerability to develop cocaine addictive behaviour.

We also observed an enhanced MSEW-induced excitability of NAc neurons in males due to the increase GluA1 and GluA1/GluA2 expression in these animals, which would lead to a higher GABAergic inhibition of VTA dopaminergic neurons. These observations sustain our hypothesis formulated in the Objective 1.

Regarding cocaine exposure, our results evidenced that only males showed increase pCREB/CREB ratio after chronic cocaine exposure. This is in accordance with our suggestion in Objective 1 of the existence of a compensatory mechanism in the males after cocaine exposure to avoid the negative effects of the drug.

This homeostatic mechanism could be to induce dynorphin synthesis and amortise the cocaine-induced dopamine release in the VTA. As consequence, the dysphoric effects in males would seem to be stronger than in females, which may be considered a protective measure against cocaine addiction. Such a hypothesis is supported by a recent study showing females to be less sensitive than males to the KOR-mediated reward-decreasing effect, due to higher VTA TH levels, which increase dopamine synthesis and protect them against the suppression of dopamine release and anhedonia (Conway et al., 2019). This hypothesis is also in agreement with the results showing that tonic dopamine levels in the NAc were not different due to sex or social stress factors (Holly et al., 2012). However, after cocaine exposure (i.p), stressed animals showed important changes of dopamine levels in NAc compared with

the non-stressed rats, but cocaine-induced dopamine elevation lasted longer in females (Holly et al., 2012).

Respect to the VTA results, our results allow to suggest that the increased cocaine-seeking in females after a chronic exposure is due to the higher GluA1, which would induce deep neuroplasticity changes than in males, understanding why females progress faster to addiction.

We also propose that males, especially those exposed to MSEW, showed enhanced excitability of the VTA dopaminergic neurons, most probably to compensate the higher GABAergic inhibition from the NAc, as mention in Objective 1.

Additionally, we observed that MSEW induced male-specific increased function of the AMPA receptor, thus explaining why MSEW only affects the acquisition of cocaine SA in males.

Due to changes in AMPA receptor function and to the higher basal excitability of VTA neurons (GluA1/GluA2), cocaine consumption would induce a higher dopamine release in males (especially MSEW males) than in females. This is in agreement with the results of *Holly* et al. (2012) in which male rats exposed to social stress showed a greater change from baseline dopamine levels in response to cocaine, in comparison with non-stressed males. However, between stressed and non-stressed females, no statistical difference was found (Holly et al., 2012).

In order to reduce the cocaine-induce higher dopamine release in the VTA, males showed decreased CREB activation (pCREB), GluA1 expression, and possibly TH synthesis, and finally dopamine firing.

Besides, our results yield that pCREB and GluA1 levels were increase in females after acquisition in comparison with the levels of males drug-experienced.

Therefore, chronic cocaine exposure induces a higher pCREB and GluA1 in females in comparison with males, leaving the AMPA receptor more susceptible to be activated, helping to understand the telescoping effect.

5. Underlying molecular changes of MSEW-induced emotional alterations in the mPFC of males and female mice.

As stated earlier, our hypothesis assumes that MSEW males have greater glutamatergic function in the NAc, triggering higher GABAergic inhibition from the NAc to the VTA. Then, males, especially those exposed to MSEW, display an enhanced excitability of VTA dopaminergic neurons to compensate this inhibition.

Normally, VTA dopaminergic projections to the PFC, inhibit glutamatergic PFC neurons (Pirot et al., 1992). Therefore, reduced dopamine firing in the VTA implies greater glutamatergic activity from the PFC to the NAc.

Therefore, we want to explore MSEW-induces changes in gene expression of AMPA receptor subunits Gria1 and Gria2 (codifies for GluA1 and GluA2, respectively). Additionally, we explore the correlation between levels of these gene and despair-like behaviour.

To achieve this, we measure the immobility time in the TST to measure despair-like behaviour in MSEW female and male mice.

Recent studies show that maternal separation in mice induces depression-like behaviours as well as a reduction of serotonin and dopamine levels in the frontal cortex (Récamier-Carballo et al., 2017). Our results show that MSEW induces a despair-like behaviour in adult male mice, whilst females seem to be resilient to this type of stress.

Animal models of depression suggest a reduced glutamate level in the PFC of depressed mice (Belin et al., 2008), as well as reduced glutamate and glutamate/glutamine levels in depressed rats (Li et al., 2008). Together with this, clinical evidence reported increased Gria2 mRNA levels in PFC of patients with major depression, and no changes in Gria1 expression (Kleinman et al., 2015). Additionally, it was reported reduced glutamate/glutamine and GABA levels in the PFC of depressed patients (Hasler et al., 2007). These previous studies are in accordance with the fact that the glutamatergic system, especially in the frontal cortex, plays a key role in the modulation of the depressive phenotype.

Our results for the gene expression of Gria1, reveal no significant differences between groups. However, results for Gria2 show that MSEW reduce Gria2 expression compared with the SN group. This result is in accordance with Ganguly et al. (2019) who reported that males maternally separated showed decreased mPFC-Gria2 expression compared to control males.

Moreover, we obtained that SN females showed decrease level of Gria2 compared with the SN males. Finally, we observed a significant reduction in the Gria2 mRNA levels of MSEW exposed male mice respect the SN males that evidences a significant mood alteration.

For this reason, we correlate levels of Gria2 with immobility time in the TST. Our results yield a negative correlation between Gria2 in the mPFC and despair-like behaviour, meaning that a decreased Gria2 correlates with increased immobility time in the TST.

We also explored the functional form of the gene, the protein levels of GluA1 and GluA2. We observed that females showed higher GluA1 and GluA2 than males, in hand with previous report of Ganguly et al. (2019) showing that females in general have higher GluA2 protein level than male mice. However, we did not observe MSEW-induced differences in the protein level of these AMPA receptor subunits (GluA1, GluA2 or GluA1/GluA2 ratio).

Epidemiologic studies showed that childhood adversity increased the risk of depression by 28.4% but also to illicit drug use by 16.5% (Anda et al., 2006). Animal studies exploring how stressful situations influence addiction-like behaviours described that acute and chronic stress alters the phosphorylation of GluA2, affecting the function of the AMPA receptor but no the GluA1/GluA2 ratio in the NAc and hippocampus (Caudal et al., 2016, 2010; Ellis et al., 2017).

This evidence could explain why we were not able to find differences in the GluA1/GluA2 ratio of MSEW male mice. It is possible that MSEW-exposed male mice showed decreased-induced phosphorylation

of GluA2, which did not modify the GluA2 levels, but altered indirectly the excitability of the AMPA receptor function in the mPFC, in accordance with our original hypothesis.

6. Sex-differences in cocaine-induced impulsivity in MSEW mice and mPFC alterations of the AMPA receptor composition after cocaine SA.

The role of personality or cognitive factors in drug abuse has been a particular topic of interest (Adinoff and Stein, 2011). Recently, impulsivity has received considerable attention due to the relevance in drug risk (Adinoff and Stein, 2011) or the development of other psychiatric disorders like depression (Dent and Isles 2014; Dalley and Ersche 2019). Because of this reason, this personality trait has been proposed as a endophenotype to investigate the underlying neurobiological mechanisms for many of impulse control disorders (Dalley and Ersche, 2019). Additionally, Brodsky et al. (2001) showed higher impulsivity and depression diagnosis in subjects with history of childhood trauma (Brodsky et al., 2001).

Studies showed that drug addicted individuals tend to show heightened impulsivity (Adinoff and Stein, 2011). This suggest the hypothesis that impulsivity may precede drug use and may increase an individual's risk of use and abuse (Adinoff and Stein, 2011).

The mPFC is a region that modulates cognitive and executive functions, including inhibitory control (Narayanan and Laubach, 2017). Clinical studies show that cocaine addiction includes poor inhibitory control for the goal-directed behaviour in the frontal cortical regions, which induce craving for the drug (Barrós-Loscertales et al., 2020; Squire, 2008).

Moreover, drug-induced impulsive behaviour could be seen as result of excessive drive for reinforcement and/or by compromised top-down, prefrontally-mediated control over these drives (Adinoff and Stein, 2011).

In line with this, immediate rewards has been related with increased activity in the VTA and the ventral striatum (Adinoff and Stein, 2011). Moreover, authors hypothesized that the observable impulsivity of a drug-addicted individual could result from excessive drive for reward or compromised control (Adinoff and Stein, 2011).

This converges in the idea that PFC and the striatum function cooperated to the reward seeking and impulse control. Therefore, we explored sex-differences in impulsivity in order to explain why females are more vulnerable to cocaine abuse than males. We also determined alterations in AMPA receptor subunits in the mPFC of mice, before and after cocaine SA.

To determine if depressed animals exposed to MSEW showed different impulsivity for cocaine, we performed the cocaine SA procedure and calculated the average of the percentage of response efficiency (indirect measure of impulsivity) along the acquisition phase.

Our results show elevated impulsivity after chronic cocaine exposure in females compared with males. In fact, SN females showed higher impulsivity than SN males after cocaine SA. This yield that SN females behaves similar (in terms of cocaine-induced impulsivity) than the MSEW males and MSEW females. Moreover, we observed elevated cocaine-induce impulsivity in MSEW males than the SN males. These

behavioural results evidenced that females, independently of the early-life stress exposure have increased impulsivity, while MSEW in males has significative consequences, increasing impulsivity for cocaine-seeking. Finally, we observed that the MSEW-increased impulsivity in males was specific for cocaine because all the groups showed similar percentages of response efficiency in the food SA.

Regarding molecular markers, Caffino et al. (2015) report that a single cocaine exposure in adolescent rats, can reduce the number of dendritic spines without any changes of GluA1 or GluA2 in the total mPFC homogenate. Our biochemical results after cocaine SA indicated a general sex effect in AMPA receptor subunits, in which females are expressing more GluA1 and GluA2 than males. Additionally, we reported elevated GluA1 in MSEW females when compared with levels of SN females and MSEW males respectively. However, in accordance with (Caffino et al., 2015), we did not find any alteration induced *per se* by the drug exposure.

Clinical studies showed a direct correlation between glutamate level in lumbar cerebrospinal fluid and impulsivity, confirming the key role of this neurotransmitter in the regulation of this personality trait (Coccaro et al., 2013). Moreover, cocaine-dependent patients showed a negative correlation between the activation of the frontoparietal network and dependence severity (Barrós-Loscertales et al., 2020).

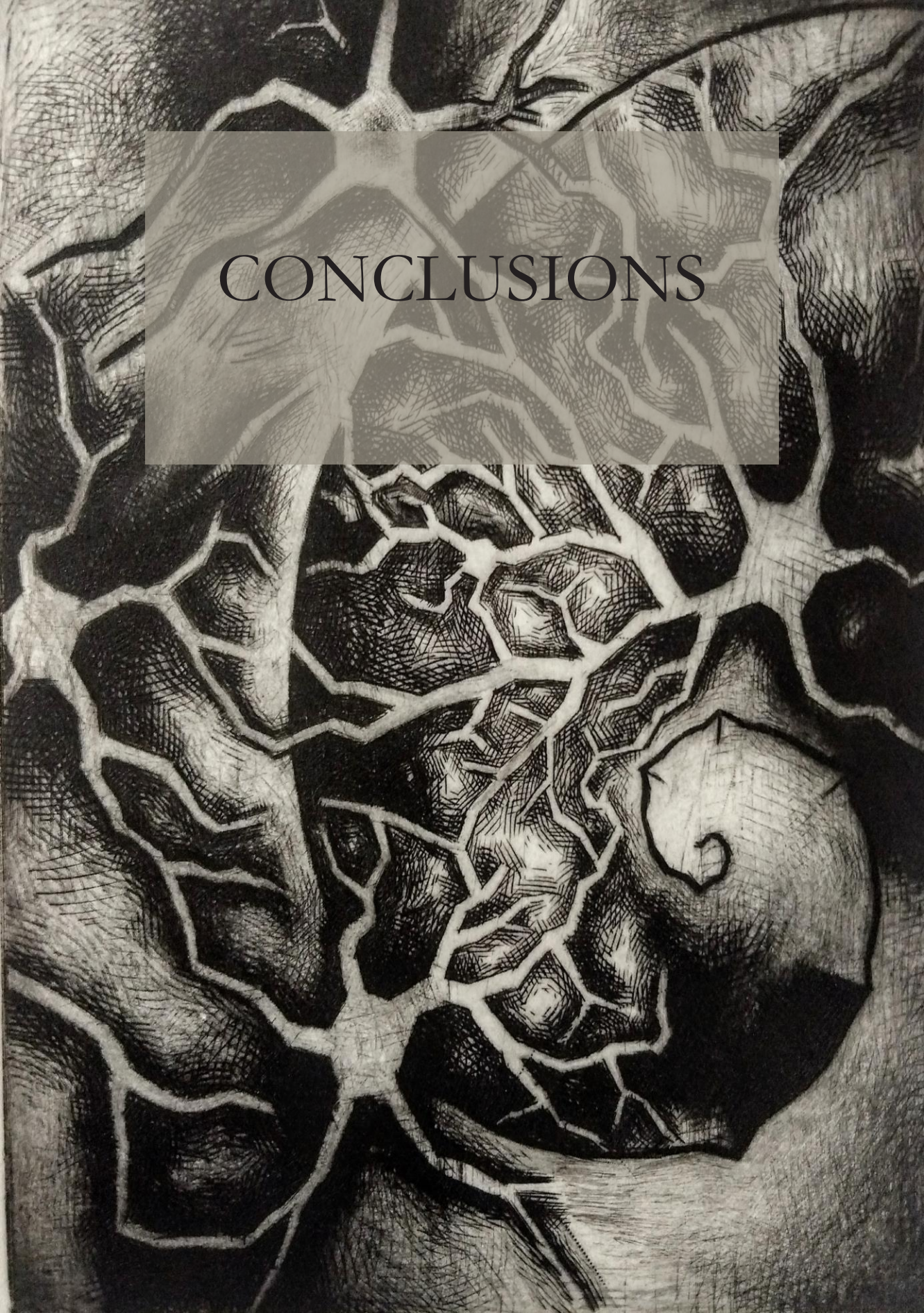
In this thesis we observed that females, the group with higher impulsivity during cocaine SA, have higher GluA1 protein expression than males. We suggest that this increased GluA1 expression, could be interpreted as a facilitated activation of the glutamatergic function in the

mPFC, explaining why females show higher impulsivity during cocaine SA than males.

Following this idea, it could be expected that MSEW males exposed to cocaine also showed an increase GluA1 level. However, as discussed above, there is a possibility that the differences in the response efficiency during the cocaine SA between the SN and the MSEW male mice can be explained by changes in the phosphorylation of GluA2 that affect the function of the AMPA receptor (Caudal et al., 2016, 2010; Ellis et al., 2017).

Why does this not happen in females? We propose that in the case of the MSEW females, the increased basal level of GluA1 could be compensating the cocaine-induced hyperphosphorylation of GluA2, avoiding further increases on impulsivity for cocaine-seeking due to MSEW.

Our hypothesis is in accordance with recent findings using mutant mice lacking GRIP1 in the mPFC (a scaffolding protein that stabilizes GluA2 at the surface) (Wickens et al., 2019). They reported that GRIP knock-out mice showed increased GluA2-containing AMPA receptors in the cell membrane, together with higher cocaine intake during the SA paradigm (Wickens et al., 2019). Moreover, Wickens et al. (2019) observed that these effects were cocaine specific and GRIP1 does not influence natural reward-seeking (Wickens et al., 2019), as we observed in the current work.



CONCLUSIONS

The main conclusions of this Doctoral Thesis can be summarized as follows:

1. The reinforcing effects of cocaine are more powerful in female mice, supporting the idea that being a female is a risk factor to develop cocaine addiction.

2. MSEW is a risk factor for cocaine addiction in males, while females seem to be more resilient to this kind of early-life stress.

3. MSEW increases despair-like behaviour, the percentage of acquisition, cocaine intake, number of infusions and nosepokes during the acquisition phase, albeit solely in male mice.

4. MSEW reduce the capacity to extinguish the cocaine-seeking behaviour in male mice, avoiding the capacity to reduce CREB expression in the NAc.

5. Females mice exhibit increased basal mPFC glutamatergic function, which potentiates impulsivity to cocaine consumption during the SA.

6. Females, independently of the early-life stress exposure have increased impulsivity in cocaine-seeking, while MSEW in males have significative consequences, increasing their impulsivity for cocaine-seeking.

7. MSEW reduce the expression of Gria2 in the mPFC of mice, a gene that correlates with despair-like behaviour.

8. GluA1 in the NAc of females, is a factor that increase vulnerability to cocaine SA but confers resilience to MSEW-induced early-life stress.

9. MSEW enhances NAc-glutamatergic function in males which increase pCREB, GluR1 and dynorphin, changes fitting with depressive-like behaviours. These alterations potentiate the inhibitory function of GABAergic neurotransmission from the NAc to the VTA, inhibiting the release of dopamine

10. Because the NAc GluA1 basal level in females are already high, they are less affected by the MSEW-induced GluA1 alteration. This higher GluA1 level in females would seem to explain why they are more vulnerable to cocaine addiction but resilient to such stress.

11. Males showed increase pCREB/CREB ratio after chronic cocaine exposure, as a mechanism to avoid the negative effects of the drug, inducing dynorphin synthesis and amortise the cocaine-induced dopamine release in the VTA. As consequence, the dysphoric effects in males would seem to be stronger than in females, which may be considered a protective measure against cocaine addiction.

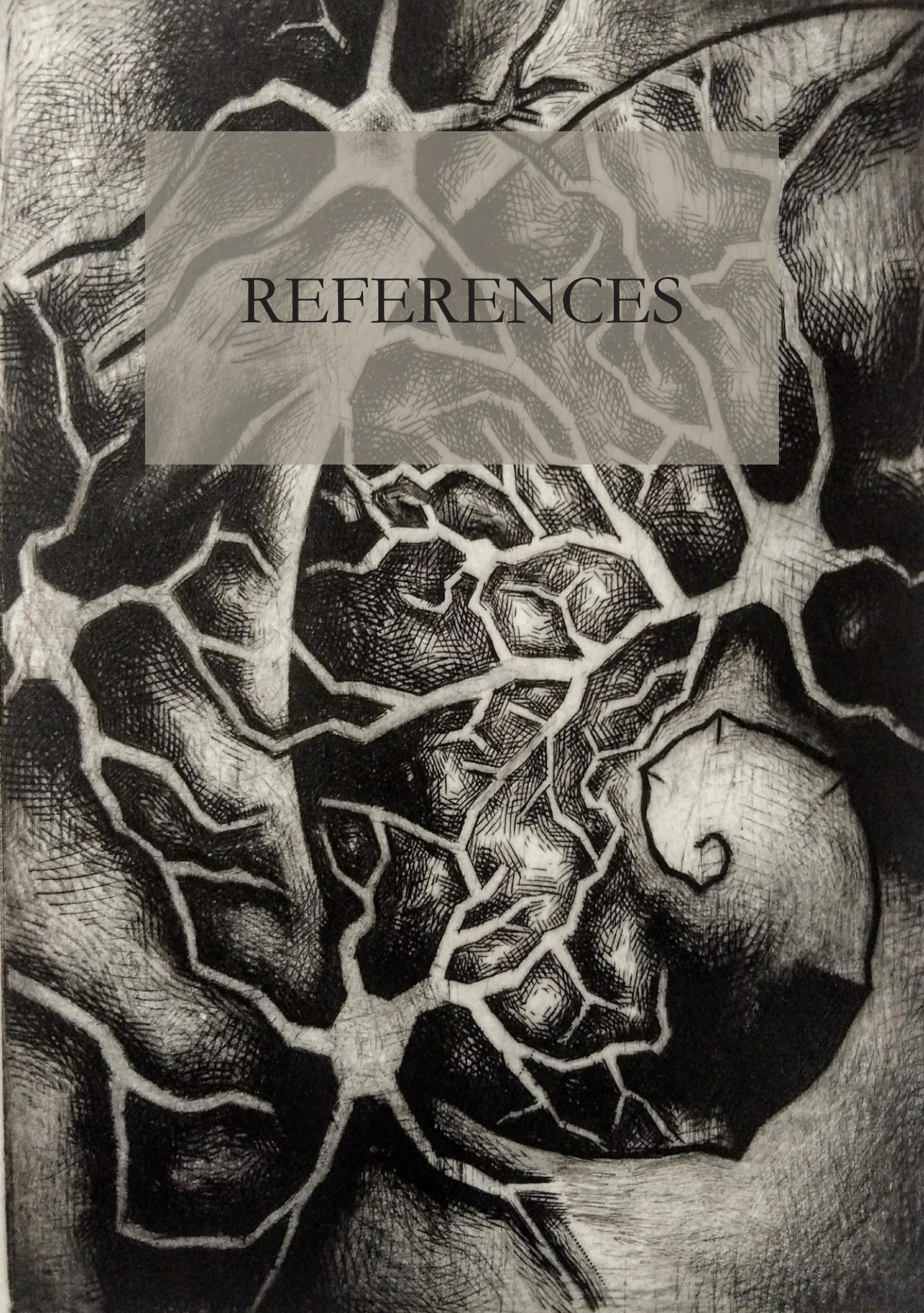
12. Due to the enhanced inhibition of the VTA dopamine neurons, MSEW males will develop depressive-like behaviour, higher

acquisition rates and tolerance to the rewarding effects of cocaine, being more susceptibility to cocaine-seeking.

13. Males exhibit higher excitability of dopaminergic neurons in the VTA, especially in the case of MSEW-exposed males, probably to compensate for the higher GABAergic inhibition from the NAc. This enhanced excitability contributes to the higher vulnerability to cocaine SA and depressive-like behaviour.

14. This thesis provides novel evidence regarding MSEW-induced glutamatergic alterations in the mesocorticolimbic system of mice, as well as novel information about sex differences in cocaine-seeking behaviour.

15. These findings could contribute to find new therapeutic targets to treat CUD.



REFERENCES

REFERENCES

Abbasi, M.A., Saeidi, M., Khademi, G., Hoseini, B.L., Moghadam, Z.E., 2015. Child maltreatment in the worldwide: A review article. Int. J. Pediatr. 3, 353–365. https://doi.org/10.22038/ijp.2015.3753

Adams, T., Rapinda, K.K., Frohlich, J.R., O'Connor, R.M., Keough, M.T., 2019. Impulsivity moderates the effect of social anxiety on in-lab alcohol craving. Addict. Behav. 97, 70–76. https://doi.org/10.1016/j.addbeh.2019.05.025

Adesnik, H., Nicoll, R.A., 2007. Conservation of Glutamate Receptor 2-Containing AMPA Receptors during Long-Term Potentiation. J. Neurosci. 27, 4598–4602. https://doi.org/10.1523/JNEUROSCI.0325-07.2007

Adinoff, B., Stein, E.A., 2011. Neuroimaging in Addiction, Neuroimaging in Addiction. Wiley-Blackwell. https://doi.org/10.1002/9781119998938

Algallal, H., Allain, F., Ndiaye, N.A., Samaha, A.N., 2019. Sex differences in cocaine self-administration behaviour under long access versus intermittent access conditions. Addict. Biol. e12809. https://doi.org/10.1111/adb.12809

Anda, R.F., Felitti, V.J., Bremner, J.D., Walker, J.D., Whitfield, C., Perry, B.D., Dube, S.R., Giles, W.H., 2006. The enduring effects of abuse and related adverse experiences in childhood: A convergence of evidence from neurobiology and epidemiology. Eur. Arch. Psychiatry Clin. Neurosci. 256, 174–186.

https://doi.org/10.1007/s00406-005-0624-4

Andrásfalvy, Bertalan K, Magee, J.C., Andrásfalvy, B K, 2004. Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones. J Physiol 559, 543–554. https://doi.org/10.1113/jphysiol.2004.065219

Anier, K., Malinovskaja, K., Pruus, K., Aonurm-Helm, A., Zharkovsky, A., Kalda, A., 2014. Maternal separation is associated with DNA methylation and behavioural changes in adult rats. Eur. Neuropsychopharmacol. 24, 459–468. https://doi.org/10.1016/j.euroneuro.2013.07.012

Anker, J.J., Gliddon, L.A., Carroll, M.E., 2008. Impulsivity on a Go/No-go task for intravenous cocaine or food in male and female rats selectively bred for high and low saccharin intake. Behav. Pharmacol. 19, 615–629. https://doi.org/10.1097/FBP.0b013e32830dc0ae

Arena, D.T., Covington, H.E., DeBold, J.F., Miczek, K.A., 2019. Persistent increase of I.V. cocaine self-administration in a subgroup of C57BL/6J male mice after social defeat stress. Psychopharmacology (Berl). 236, 2027–2037. https://doi.org/10.1007/s00213-019-05191-6

Argilli, E., Sibley, D.R., Malenka, R.C., England, P.M., Bonci, A., 2008. Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area. J. Neurosci. 28, 9092–100. https://doi.org/10.1523/JNEUROSCI.1001-08.2008

Back, S.E., Hartwell, K., DeSantis, S.M., Saladin, M., McRae-Clark, A.L., Price, K.L., Moran-Santa Maria, M.M., Baker, N.L., Spratt, E., Kreek, M.J., Brady, K.T., 2010. Reactivity to laboratory stress provocation predicts relapse to cocaine. Drug Alcohol Depend. 106, 21–27. https://doi.org/10.1016/j.drugalcdep.2009.07.016

Bäckström, P., Hyytiä, P., 2006. Ionotropic and Metabotropic Glutamate Receptor Antagonism Attenuates Cue-Induced Cocaine Seeking. Neuropsychopharmacology 31, 778–786. https://doi.org/10.1038/sj.npp.1300845

Bäckström, P., Hyytiä, P., 2003. Attenuation of cocaine-seeking behaviour by the AMPA/kainate receptor antagonist CNQX in rats. Psychopharmacology (Berl). 166, 69–76. https://doi.org/10.1007/s00213-002-1312-y

Bagley, J.R., Adams, J., Bozadjian, R. V., Bubalo, L., Kippin, T.E., 2019a. Strain differences in maternal neuroendocrine and behavioral responses to stress and the relation to offspring cocaine responsiveness. Int. J. Dev. Neurosci. 78, 130–138. https://doi.org/10.1016/j.ijdevneu.2019.06.009

Bagley, J.R., Szumlinski, K.K., Kippin, T.E., 2019b. Discovery of early life stress interacting and sex-specific quantitative trait loci impacting cocaine responsiveness. Br. J. Pharmacol. 176, 4159–4172. https://doi.org/10.1111/bph.14661

Bannon, M.J., Pruetz, B., Manning-Bog, A.B., Whitty, C.J., Michelhaugh, S.K., Sacchetti, P., Granneman, J.G., Mash, D.C., Schmidt, C.J., 2002. Decreased expression of the transcription

factor NURR1 in dopamine neurons of cocaine abusers. Proc. Natl. Acad. Sci. U. S. A. 99, 6382–5. https://doi.org/10.1073/pnas.092654299

Baracz, S.J., Everett, N.A., Cornish, J.L., 2020. The impact of early life stress on the central oxytocin system and susceptibility for drug addiction: Applicability of oxytocin as a pharmacotherapy. Neurosci. Biobehav. Rev. 110, 114–132. https://doi.org/10.1016/j.neubiorev.2018.08.014

Barkus, C., Feyder, M., Graybeal, C., Wright, T., Wiedholz, L., Izquierdo, A., Kiselycznyk, C., Schmitt, W., Sanderson, D.J., Rawlins, J.N.P., Saksida, L.M., Bussey, T.J., Sprengel, R., Bannerman, D., Holmes, A., 2012. Do GluA1 knockout mice exhibit behavioral abnormalities relevant to the negative or cognitive symptoms of schizophrenia and schizoaffective disorder? Neuropharmacology 62, 1263–1272. https://doi.org/10.1016/J.NEUROPHARM.2011.06.005

Barria, A., 2009. Silent Synapse, Encyclopedia of Neuroscience. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-29678-2

Barrós-Loscertales, A., Costumero, V., Rosell-Negre, P., Fuentes-Claramonte, P., Llopis-Llacer, J.-J., Bustamante, J.C., 2020. Motivational factors modulate left frontoparietal network during cognitive control in cocaine addiction. Addict. Biol. 25, e12820. https://doi.org/10.1111/adb.12820

Barrot, M., Olivier, J.D.A., Perrotti, L.I., DiLeone, R.J., Berton, O.,

Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C., Zachariou, V., Nestler, E.J., 2002. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc. Natl. Acad. Sci. 99, 11435–11440. https://doi.org/10.1073/pnas.172091899

Becker, J.B., McClellan, M.L., Reed, B.G., 2017. Sex differences, gender and addiction. J. Neurosci. Res. 95, 136–147. https://doi.org/10.1002/jnr.23963

Belin, D., Mar, A.C., Dalley, J.W., Robbins, T.W., Everitt, B.J., 2008. High impulsivity predicts the switch to compulsive cocaine-taking. Science 320, 1352–5. https://doi.org/10.1126/science.1158136

Bian, Y., Yang, L., Wang, Z., Wang, Q., Zeng, L., Xu, G., 2015. Repeated three-hour maternal separation induces depression-like behavior and affects the expression of hippocampal plasticity-related proteins in C57BL/6N mice. Neural Plast. 2015, 5–11. https://doi.org/10.1155/2015/627837

Birnie, M.T., Kooiker, C.L., Short, A.K., Bolton, J.L., Chen, Y., Baram, T.Z., 2020. Plasticity of the Reward Circuitry After Early-Life Adversity: Mechanisms and Significance. Biol. Psychiatry 87, 875–884. https://doi.org/10.1016/j.biopsych.2019.12.018

Bissonette, G.B., Roesch, M.R., 2016. Development and function of the midbrain dopamine system: what we know and what we need to. Genes. Brain. Behav. 15, 62–73. https://doi.org/10.1111/gbb.12257

Boudreau, A.C., Wolf, M.E., 2005. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J. Neurosci. 25, 9144–51. https://doi.org/10.1523/JNEUROSCI.2252-05.2005

Bowers, M.S., Chen, B.T., Bonci, A., 2010. AMPA receptor synaptic plasticity induced by psychostimulants: the past, present, and therapeutic future. Neuron 67, 11–24. https://doi.org/10.1016/j.neuron.2010.06.004

Brodsky, B.S., Oquendo, M., Ellis, S.P., Haas, G.L., Malone, K.M., Mann, J.J., 2001. The relationship of childhood abuse to impulsivity and suicidal behavior in adults with major depression. Am. J. Psychiatry 158, 1871–1877. https://doi.org/10.1176/appi.ajp.158.11.1871

Brown, T.E., Lee, B.R., Mu, P., Ferguson, D., Dietz, D., Ohnishi, Y.N., Lin, Y., Suska, A., Ishikawa, M., Huang, Y.H., Shen, H., Kalivas, P.W., Sorg, B.A., Zukin, R.S., Nestler, E.J., Dong, Y., Schluter, O.M., 2011. A Silent Synapse-Based Mechanism for Cocaine-Induced Locomotor Sensitization. J. Neurosci. 31, 8163–8174. https://doi.org/10.1523/JNEUROSCI.0016-11.2011

Butelman, E.R., Chen, C.Y., Conybeare, R.A., Brown, K.G., Fry, R.S., Kimani, R., da Rosa, J.C., Ott, J., Kreek, M.J., 2019. Are Trait Impulsivity and Exposure to Cannabis or Alcohol Associated With the Age of Trajectory of Cocaine Use? A Gender-Specific Dimensional Analysis in Humans With Cocaine Dependence Diagnosis. Exp. Clin. Psychopharmacol. 28(3), 317–327.

https://doi.org/10.1037/pha0000314

Caffino, L., Giannotti, G., Malpighi, C., Racagni, G., Fumagalli, F., 2015. Short-term withdrawal from developmental exposure to cocaine activates the glucocorticoid receptor and alters spine dynamics. Eur. Neuropsychopharmacol. 25, 1832–1841. https://doi.org/10.1016/j.euroneuro.2015.05.002

Caine, S.B., Bowen, C.A., Yu, G., Zuzga, D., Negus, S.S., Mello, N.K., 2004. Effect of gonadectomy and gonadal hormone replacement on cocaine self-administration in female and male rats. Neuropsychopharmacology 29, 929–42. https://doi.org/10.1038/sj.npp.1300387

Cardenas-Perez, R.E., Fuentes-Mera, L., de la Garza, A.L., Torre-Villalvazo, I., Reyes-Castro, L.A., Rodriguez-Rocha, H., Garcia-Garcia, A., Corona-Castillo, J.C., Tovar, A.R., Zambrano, E., Ortiz-Lopez, R., Saville, J., Fuller, M., Camacho, A., 2018. Maternal overnutrition by hypercaloric diets programs hypothalamic mitochondrial fusion and metabolic dysfunction in rat male offspring. Nutr. Metab. (Lond). 15, 38. https://doi.org/10.1186/s12986-018-0279-6

Carlezon, W.A., Boundy, V.A., Haile, C.N., Lane, S.B., Kalb, R.G., Neve, R.L., Nestler, E.J., 1997. Sensitization to morphine induced by viral-mediated gene transfer. Science (80-.). 277, 812–814. https://doi.org/10.1126/science.277.5327.812

Carlezon, W.A., Thome, J., Olson, V.G., Lane-Ladd, S.B., Brodkin, E.S., Hiroi, N., Duman, R.S., Neve, R.L., Nestler, E.J., 1998.

Regulation of Cocaine Reward by CREB. Science (80-.). 282, 2272–2275. https://doi.org/10.1126/science.282.5397.2272

Carter, M., Shieh, J., 2015. Guide to Research Techniques in Neuroscience, Guide to Research Techniques in Neuroscience. Elsevier. https://doi.org/10.1016/B978-0-12-800511-8.00002-2

Castro-Zavala, A., Martín-Sánchez, A., Valverde, O., 2020. Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice. Eur. Neuropsychopharmacol. 32, 12–24. https://doi.org/10.1016/j.euroneuro.2019.12.112

Caudal, D., Godsil, B.P., Mailliet, F., Bergerot, D., Jay, T.M., 2010. Acute Stress Induces Contrasting Changes in AMPA Receptor Subunit Phosphorylation within the Prefrontal Cortex, Amygdala and Hippocampus. PLoS One 5, e15282. https://doi.org/10.1371/journal.pone.0015282

Caudal, D., Rame, M., Jay, T.M., Godsil, B.P., 2016. Dynamic Regulation of AMPAR Phosphorylation In Vivo Following Acute Behavioral Stress. Cell. Mol. Neurobiol. 36, 1331–1342. https://doi.org/10.1007/s10571-016-0332-9

Center on the developing Child, n.d. Violence Info – Child maltreatment [WWW Document]. URL http://apps.who.int/violence-info/child-maltreatment (accessed 3.12.20).

Chao, S.Z., Ariano, M.A., Peterson, D.A., Wolf, M.E., 2002. D1 dopamine receptor stimulation increases GluR1 surface

expression in nucleus accumbens neurons. J. Neurochem. 83, 704–712. https://doi.org/10.1046/j.1471-4159.2002.01164.x

Charney, D.S., Manji, H.K., 2004. Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. Sci. STKE 2004, re5. https://doi.org/10.1126/stke.2252004re5

Chen, A.C.H., Shirayama, Y., Shin, K.H., Neve, R.L., Duman, R.S., 2001. Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. Biol. Psychiatry 49, 753–762. https://doi.org/10.1016/S0006-3223(00)01114-8

Chen, K., Kandel, D., 2002. Relationship between extent of cocaine use and dependence among adolescents and adults in the United States. Drug Alcohol Depend. 68, 65–85. https://doi.org/10.1016/S0376-8716(02)00086-8

Childhelp, 2020. Child abuse statistics. [WWW Document]. URL https://www.childhelp.org/child-abuse-statistics/ (accessed 5.27.20).

Children's Bureau, 2018. Child Maltreatment 2018.

Chocyk, A., Bobula, B., Dudys, D., Przyborowska, A., Majcher-Maślanka, I., Hess, G., Wedzony, K., Wędzony, K., 2013. Early-life stress affects the structural and functional plasticity of the medial prefrontal cortex in adolescent rats. Eur. J. Neurosci. 38, 2089–2107. https://doi.org/10.1111/ejn.12208

Choi, K.H., Edwards, S., Graham, D.L., Larson, E.B., Whisler, K.N., Simmons, D., Friedman, A.K., Walsh, J.J., Rahman, Z., Monteggia, L.M., Eisch, A.J., Neve, R.L., Nestler, E.J., Han, M.-H., Self, D.W., 2011. Reinforcement-Related Regulation of AMPA Glutamate Receptor Subunits in the Ventral Tegmental Area Enhances Motivation for Cocaine. J. Neurosci. 31, 7927–7937. https://doi.org/10.1523/JNEUROSCI.6014-10.2011

Chourbaji, S., Vogt, M.A., Fumagalli, F., Sohr, R., Frasca, A., Brandwein, C., Hörtnagl, H., Riva, M.A., Sprengel, R., Gass, P., 2008. AMPA receptor subunit 1 (GluR-A) knockout mice model the glutamate hypothesis of depression. FASEB J. 22, 3129–34. https://doi.org/10.1096/fj.08-106450

Churchill, L., Swanson, C.J., Urbina, M., Kalivas, P.W., 1999. Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. J. Neurochem. 72, 2397–403.

Clark, L., Robbins, T.W., Ersche, K.D., Sahakian, B.J., 2006. Reflection Impulsivity in Current and Former Substance Users. Biol. Psychiatry 60, 515–522. https://doi.org/10.1016/j.biopsych.2005.11.007

Coccaro, E.F., Lee, R., Vezina, P., 2013. Cerebrospinal fluid glutamate concentration correlates with impulsive aggression in human subjects. J. Psychiatr. Res. 47, 1247–1253. https://doi.org/10.1016/j.jpsychires.2013.05.001

Cohen, J.R., Menon, S. V., Shorey, R.C., Le, V.D., Temple, J.R., 2017.

The distal consequences of physical and emotional neglect in emerging adults: A person-centered, multi-wave, longitudinal study. Child Abus. Negl. 63, 151–161. https://doi.org/10.1016/j.chiabu.2016.11.030

Conrad, K.L., Tseng, K.Y., Uejima, J.L., Reimers, J.M., Heng, L.-J., Shaham, Y., Marinelli, M., Wolf, M.E., 2008. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature 454, 118–21. https://doi.org/10.1038/nature06995

Conway, S.M., Puttick, D., Russell, S., Potter, D., Roitman, M.F., Chartoff, E.H., 2019. Females are less sensitive than males to the motivational- and dopamine-suppressing effects of kappa opioid receptor activation. Neuropharmacology 146, 231–241. https://doi.org/10.1016/j.neuropharm.2018.12.002

Corruble, E., Benyamina, A., Bayle, F., Falissard, B., Hardy, P., 2003. Understanding impulsivity in severe depression? A psychometrical contribution. Prog. Neuro-Psychopharmacology Biol. Psychiatry 27, 829–833. https://doi.org/10.1016/S0278-5846(03)00115-5

Covington, H.E., Maze, I., Sun, H., Bomze, H.M., DeMaio, K.D., Wu, E.Y., Dietz, D.M., Lobo, M.K., Ghose, S., Mouzon, E., Neve, R.L., Tamminga, C.A., Nestler, E.J., 2011. A Role for Repressive Histone Methylation in Cocaine-Induced Vulnerability to Stress. Neuron 71, 656–670. https://doi.org/10.1016/j.neuron.2011.06.007

Cryan, J.F., O'Leary, O.F., 2010. Neuroscience. A glutamate pathway to

faster-acting antidepressants? Science 329, 913–4. https://doi.org/10.1126/science.1194313

Cryan, J.F., Slattery, D.A., 2010. GABAB receptors and depression. Current status. Adv. Pharmacol. 58, 427–51. https://doi.org/10.1016/S1054-3589(10)58016-5

Cummings, J.A., Gowl, B.A., Westenbroek, C., Clinton, S.M., Akil, H., Becker, J.B., 2011. Effects of a selectively bred novelty-seeking phenotype on the motivation to take cocaine in male and female rats. Biol. Sex Differ. 2, 3. https://doi.org/10.1186/2042-6410-2-3

D'Ascenzo, M., Podda, M.V., Grassi, C., 2014. The role of D-serine as co-agonist of NMDA receptors in the nucleus accumbens: relevance to cocaine addiction. Front. Synaptic Neurosci. 6, 16. https://doi.org/10.3389/fnsyn.2014.00016

D'Sa, C., Duman, R.S., 2002. Antidepressants and neuroplasticity. Bipolar Disord. 4, 183–94. https://doi.org/10.1034/j.1399-5618.2002.01203.x

Dalley, J.W., Ersche, K.D., 2019. Neural circuitry and mechanisms of waiting impulsivity: relevance to addiction. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 374, 20180145. https://doi.org/10.1098/rstb.2018.0145

Dalley, J.W., Everitt, B.J., Robbins, T.W., 2011. Impulsivity, compulsivity, and top-down cognitive control. Neuron 69, 680–94. https://doi.org/10.1016/j.neuron.2011.01.020

Dalley, J.W., Roiser, J.P., 2012. Dopamine, serotonin and impulsivity. Neuroscience 215, 42–58. https://doi.org/10.1016/j.neuroscience.2012.03.065

Daskalakis, N.P., Bagot, R.C., Parker, K.J., Vinkers, C.H., de Kloet, E.R., 2013. The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 38, 1858–1873. https://doi.org/10.1016/j.psyneuen.2013.06.008

Davis, B.A., Clinton, S.M., Akil, H., Becker, J.B., 2008. The effects of novelty-seeking phenotypes and sex differences on acquisition of cocaine self-administration in selectively bred High-Responder and Low-Responder rats. Pharmacol. Biochem. Behav. 90, 331–338. https://doi.org/10.1016/j.pbb.2008.03.008

De Rasmo, D., Signorile, A., Roca, E., Papa, S., 2009. cAMP response element-binding protein (CREB) is imported into mitochondria and promotes protein synthesis. FEBS J. 276, 4325–4333. https://doi.org/10.1111/j.1742-4658.2009.07133.x

Dent, C.L., Isles, A.R., 2014. An overview of measuring impulsive behavior in mice. Curr. Protoc. Mouse Biol. 4, 35–45. https://doi.org/10.1002/9780470942390.mo140015

Dixon, C.I., Walker, S.E., Swinny, J., Belelli, D., Lambert, J.J., King, S.L., Stephens, D.N., 2019. Early-life stress influences acute and sensitized responses of adult mice to cocaine by interacting with GABA A 2 receptor expression. Behav. Pharmacol. 30, 272–281. https://doi.org/10.1097/FBP.0000000000000466

Doan, L., Manders, T., Wang, J., 2015. Neuroplasticity underlying the comorbidity of pain and depression. Neural Plast. 2015, 504691. https://doi.org/10.1155/2015/504691

Doncheck, E.M., Liddiard, G.T., Konrath, C.D., Liu, X., Yu, L., Urbanik, L.A., Herbst, M.R., DeBaker, M.C., Raddatz, N., Van Newenhizen, E.C., Mathy, J., Gilmartin, M.R., Liu, Q.-S., Hillard, C.J., Mantsch, J.R., 2020. Sex, stress, and prefrontal cortex: influence of biological sex on stress-promoted cocaine seeking. Neuropsychopharmacology. https://doi.org/10.1038/s41386-020-0674-3

Dong, Y., Nestler, E.J., 2014. The neural rejuvenation hypothesis of cocaine addiction. Trends Pharmacol. Sci. 35, 374–83. https://doi.org/10.1016/j.tips.2014.05.005

Dowlatshahi, D., MacQueen, G.M., Wang, J.F., Young, L.T., 1998. Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. Lancet 352, 1754–1755. https://doi.org/10.1016/s0140-6736(05)79827-5

Doyle, S.E., Ramôa, C., Garber, G., Newman, J., Toor, Z., Lynch, W.J., 2014. A Shift in the Role of Glutamatergic Signaling in the Nucleus Accumbens Core With the Development of an Addicted Phenotype. Biol. Psychiatry 76, 810–815. https://doi.org/10.1016/j.biopsych.2014.02.005

Dube, S.R., Felitti, V.J., Dong, M., Chapman, D.P., Giles, W.H., Anda, R.F., 2003a. Childhood abuse, neglect, and household dysfunction and the risk of illicit drug use: the adverse childhood experiences

study. Pediatrics 111, 564–72. https://doi.org/10.1542/peds.111.3.564

Dube, S.R., Felitti, V.J., Dong, M., Giles, W.H., Anda, R.F., 2003b. The impact of adverse childhood experiences on health problems: evidence from four birth cohorts dating back to 1900. Prev. Med. (Baltim). 37, 268–77. https://doi.org/10.1016/s0091-7435(03)00123-3

Ducci, F., Roy, A., Shen, P.-H., Yuan, Q., Yuan, N.P., Hodgkinson, C.A., Goldman, L.R., Goldman, D., 2009. Association of substance use disorders with childhood trauma but not African genetic heritage in an African American cohort. Am. J. Psychiatry 166, 1031–40. https://doi.org/10.1176/appi.ajp.2009.08071068

Duman, R.S., Monteggia, L.M., 2006. A neurotrophic model for stress-related mood disorders. Biol. Psychiatry 59, 1116–27. https://doi.org/10.1016/j.biopsych.2006.02.013

Duric, V., Banasr, M., Stockmeier, C.A., Simen, A.A., Newton, S.S., Overholser, J.C., Jurjus, G.J., Dieter, L., Duman, R.S., 2013. Altered expression of synapse and glutamate related genes in post-mortem hippocampus of depressed subjects. Int. J. Neuropsychopharmacol. 16, 69–82. https://doi.org/10.1017/S1461145712000016

Dutta, S., Sengupta, P., 2016. Men and mice: Relating their ages. Life Sci. 152, 244–8. https://doi.org/10.1016/j.lfs.2015.10.025

Eagle, A., Al Masraf, B., Robison, A.J., 2019. Transcriptional and

Epigenetic Regulation of Reward Circuitry in Drug Addiction, in: Neural Mechanisms of Addiction. Elsevier, pp. 23–34. https://doi.org/10.1016/B978-0-12-812202-0.00003-8

Eisch, A.J., Bolaños, C.A., De Wit, J., Simonak, R.D., Pudiak, C.M., Barrot, M., Verhaagen, J., Nestler, E.J., 2003. Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: A role in depression. Biol. Psychiatry 54, 994–1005. https://doi.org/10.1016/j.biopsych.2003.08.003

Ellis, A.S., Fosnocht, A.Q., Lucerne, K.E., Briand, L.A., 2017. Disruption of GluA2 phosphorylation potentiates stress responsivity. Behav. Brain Res. 333, 83–89. https://doi.org/10.1016/j.bbr.2017.06.046

EMCDDA, 2019. European Drug Report 2019: Trends and Developments, European Union Publications Office. https://doi.org/10.1097/JSM.0b013e31802b4fda

Enoch, M.-A., 2011. The role of early life stress as a predictor for alcohol and drug dependence. Psychopharmacology (Berl). 214, 17–31. https://doi.org/10.1007/s00213-010-1916-6

Evanz, R., 2017. What is childhood and what do we mean by "young person"? [WWW Document]. IPPF. URL https://www.ippf.org/resource/what-childhood-and-what-do-we-mean-young-person (accessed 3.11.20).

Fagiolini, M., Hensch, T.K., 2000. Inhibitory threshold for critical-period activation in primary visual cortex. Nature 404, 183–186.

https://doi.org/10.1038/35004582

Fattore, L., Piras, G., Corda, M.G., Giorgi, O., 2009. The Roman High- and Low-Avoidance Rat Lines Differ in the Acquisition, Maintenance, Extinction, and Reinstatement of Intravenous Cocaine Self-Administration. Neuropsychopharmacology 34, 1091–1101. https://doi.org/10.1038/npp.2008.43

Fosnocht, A.Q., Lucerne, K.E., Ellis, A.S., Olimpo, N.A., Briand, L.A., 2019. Adolescent social isolation increases cocaine seeking in male and female mice. Behav. Brain Res. 359, 589–596. https://doi.org/10.1016/j.bbr.2018.10.007

Frank, D., Zlotnik, A., Kofman, O., Grinshpun, J., Severynovska, O., Brotfain, E., Kut, R., Natanel, D., Melamed, I., Boyko, M., 2019. Early life stress induces submissive behavior in adult rats. Behav. Brain Res. 372. https://doi.org/10.1016/j.bbr.2019.112025

Ganguly, P., Honeycutt, J.A., Rowe, J.R., Demaestri, C., Brenhouse, H.C., 2019. Effects of early life stress on cocaine conditioning and AMPA receptor composition are sex-specific and driven by TNF. Brain. Behav. Immun. 78, 41–51. https://doi.org/10.1016/j.bbi.2019.01.006

Gao, C., Wolf, M.E., 2007. Dopamine alters AMPA receptor synaptic expression and subunit composition in dopamine neurons of the ventral tegmental area cultured with prefrontal cortex neurons. J. Neurosci. 27, 14275–14285. https://doi.org/10.1523/JNEUROSCI.2925-07.2007

García-Marchena, N., Barrera, M., Mestre-Pintó, J.I., Araos, P., Serrano, A., Pérez-Mañá, C., Papaseit, E., Fonseca, F., Ruiz, J.J., Rodríguez de Fonseca, F., Farré, M., Pavón, F.J., Torrens, M., 2019. Inflammatory mediators and dual depression: Potential biomarkers in plasma of primary and substance-induced major depression in cocaine and alcohol use disorders. PLoS One 14, e0213791. https://doi.org/10.1371/journal.pone.0213791

Gass, J.T., Olive, M.F., 2008. Glutamatergic substrates of drug addiction and alcoholism. Biochem. Pharmacol. 75, 218–265. https://doi.org/10.1016/j.bcp.2007.06.039

GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet (London, England) 392, 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7

George, E.D., Bordner, K.A., Elwafi, H.M., Simen, A.A., 2010. Maternal separation with early weaning: a novel mouse model of early life neglect. BMC Neurosci. 11, 123. https://doi.org/10.1186/1471-2202-11-123

Giacometti, L.L., Barker, J.M., 2020. Sex differences in the glutamate system: Implications for addiction. Neurosci. Biobehav. Rev. 113, 157–168. https://doi.org/10.1016/j.neubiorev.2020.03.010

Gilbertson, M.W., Gilbertson, M.W., Shenton, M.E., Shenton, M.E.,

Ciszewski, A., Ciszewski, A., Kasai, K., Kasai, K., Lasko, N.B., Lasko, N.B., Orr, S.P., Orr, S.P., Pitman, R.K., Pitman, R.K., 2002. Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. Nat. Neurosci. 5, 1242–1247.

Giorgi, O., Corda, M.G., Fernández-Teruel, A., 2019. A Genetic Model of Impulsivity, Vulnerability to Drug Abuse and Schizophrenia-Relevant Symptoms With Translational Potential: The Roman High- vs. Low-Avoidance Rats. Front. Behav. Neurosci. 13, 145. https://doi.org/10.3389/fnbeh.2019.00145

Giorgi, O., Piras, G., Lecca, D., Corda, M.G., 2005. Behavioural effects of acute and repeated cocaine treatments: A comparative study in sensitisation-prone RHA rats and their sensitisation-resistant RLA counterparts. Psychopharmacology (Berl). 180, 530–538. https://doi.org/10.1007/s00213-005-2177-7

Goffer, Y., Xu, D., Eberle, S.E., D'amour, J., Lee, M., Tukey, D., Froemke, R.C., Ziff, E.B., Wang, J., 2013. Calcium-Permeable AMPA Receptors in the Nucleus Accumbens Regulate Depression-Like Behaviors in the Chronic Neuropathic Pain State. J. Neurosci. 33, 19034–19044. https://doi.org/10.1523/JNEUROSCI.2454-13.2013

Gogas, K.R., Lechner, S.M., Markison, S., Williams, J.P., McCarthy, W., Grigoriadis, D.E., Foster, A.C., 2007. 6.04 - Anxiety, in: Comprehensive Medicinal Chemistry II. Elsevier, pp. 85–115. https://doi.org/10.1016/B0-08-045044-X/00164-4

Goodwill, H.L., Manzano-Nieves, G., Gallo, M., Lee, H.-I., Oyerinde,

E., Serre, T., Bath, K.G., 2019. Early life stress leads to sex differences in development of depressive-like outcomes in a mouse model. Neuropsychopharmacology 44, 711–720. https://doi.org/10.1038/s41386-018-0195-5

Gorka, A.X., Hanson, J.L., Radtke, S.R., Hariri, A.R., 2014. Reduced hippocampal and medial prefrontal gray matter mediate the association between reported childhood maltreatment and trait anxiety in adulthood and predict sensitivity to future life stress. Biol. Mood Anxiety Disord. 4, 12. https://doi.org/10.1186/2045-5380-4-12

Gracia-Rubio, I., 2016. Neurobiological links between depression and drug dependence. TDX (Tesis Dr. en Xarxa). Universitat Pompeu Fabra.

Gracia-Rubio, I., Martinez-Laorden, E., Moscoso-Castro, M., Milanés, V., Laorden, L., Valverde, O., 2016a. Maternal Separation Impairs Cocaine-Induced Behavioural Sensitization in Adolescent Mice. PLoS One 11, e0167483. https://doi.org/10.1371/journal.pone.0167483

Gracia-Rubio, I., Moscoso-Castro, M., Pozo, O.J., Marcos, J., Nadal, R., Valverde, O., 2016b. Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 65, 104–17. https://doi.org/10.1016/j.pnpbp.2015.09.003

Granö, N., Keltikangas-Järvinen, L., Kouvonen, A., Virtanen, M., Elovainio, M., Vahtera, J., Kivimäki, M., 2007. Impulsivity as a

predictor of newly diagnosed depression. Scand. J. Psychol. 48, 173–9. https://doi.org/10.1111/j.1467-9450.2007.00566.x

Haas, A.L., Peters, R.H., 2000. Development of substance abuse problems among drug-involved offenders: evidence for the telescoping effect. J. Subst. Abuse 12, 241–253. https://doi.org/10.1016/S0899-3289(00)00053-5

Hartley, C.A., Lee, F.S., 2015. Sensitive Periods in Affective Development: Nonlinear Maturation of Fear Learning. Neuropsychopharmacology 40, 50–60. https://doi.org/10.1038/npp.2014.179

Hasler, G., Van Der Veen, J.W., Tumonis, T., Meyers, N., Shen, J., Drevets, W.C., 2007. Reduced prefrontal glutamate/glutamine and γ-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. Arch. Gen. Psychiatry 64, 193–200. https://doi.org/10.1001/archpsyc.64.2.193

Hemby, S., Tang, W., Muly, E., Kuhar, M., Howell, L., Mash, D., 2005. Cocaine-induced alterations in nucleus accumbens ionotropic glutamate receptor subunits in human and non-human primates. J. Neurochem. 95, 1785–93. https://doi.org/10.1111/j.1471-4159.2005.03517.x

Heshmati, M., 2009. Cocaine-Induced LTP in the Ventral Tegmental Area: New Insights Into Mechanism and Time Course Illuminate the Cellular Substrates of Addiction. J Neurophysiol 101, 2735–2737. https://doi.org/10.1152/jn.00127.2009

Hillis, S., Mercy, J., Amobi, A., Kress, H., 2016. Global Prevalence of Past-year Violence Against Children: A Systematic Review and Minimum Estimates. Pediatrics 137, e20154079. https://doi.org/10.1542/peds.2015-4079

Holly, E.N., Shimamoto, A., Debold, J.F., Miczek, K.A., 2012. Sex differences in behavioral and neural cross-sensitization and escalated cocaine taking as a result of episodic social defeat stress in rats. Psychopharmacology (Berl). 224, 179–188. https://doi.org/10.1007/s00213-012-2846-2

Houck, M.M., Siegel, J.A., 2015. Illicit Drugs, in: Fundamentals of Forensic Science. Elsevier, pp. 315–352. https://doi.org/10.1016/b978-0-12-800037-3.00013-3

Houeto, J.L., Magnard, R., Dalley, J.W., Belin, D., Carnicella, S., 2016. Trait impulsivity and anhedonia: Two gateways for the development of impulse control disorders in Parkinson's disease? Front. Psychiatry 7, 91. https://doi.org/10.3389/fpsyt.2016.00091

Hsu, F.-C., Zhang, G.-J., Raol, Y.S.H., Valentino, R.J., Coulter, D.A., Brooks-Kayal, A.R., 2003. Repeated neonatal handling with maternal separation permanently alters hippocampal GABAA receptors and behavioral stress responses. Proc. Natl. Acad. Sci. U. S. A. 100, 12213–8. https://doi.org/10.1073/pnas.2131679100

Hu, M., Becker, J.B., 2003. Effects of sex and estrogen on behavioral sensitization to cocaine in rats. J. Neurosci. 23, 693–9.

Huang, Y.H., Lin, Y., Mu, P., Lee, B.R., Brown, T.E., Wayman, G., Marie, H., Liu, W., Yan, Z., Sorg, B. a, Schlüter, O.M., Zukin, R.S., Dong, Y., 2009. In vivo cocaine experience generates silent synapses. Neuron 63, 40–47. https://doi.org/10.1016/j.neuron.2009.06.007

Hummel, M., Unterwald, E.M., 2002. D1 dopamine receptor: A putative neurochemical and behavioral link to cocaine action. J. Cell. Physiol. 191, 17–27. https://doi.org/10.1002/jcp.10078

Hurd, Y.L., Herkenham, M., 1993. Molecular alterations in the neostriatum of human cocaine addicts. Synapse 13, 357–369. https://doi.org/10.1002/syn.890130408

Hyman, S.M., Paliwal, P., Chaplin, T.M., Mazure, C.M., Rounsaville, B.J., Sinha, R., 2008. Severity of childhood trauma is predictive of cocaine relapse outcomes in women but not men. Drug Alcohol Depend. 92, 208–216. https://doi.org/10.1016/j.drugalcdep.2007.08.006

Hynes, T.J., Thomas, C.S., Zumbusch, A.S., Samson, A., Petriman, I., Mrdja, U., Orr, A., Cutts, E., Ruzindana, B.G., Hazari, A., Zjadewicz, M., Lovic, V., 2018. Early life adversity potentiates expression of addiction-related traits. Prog. Neuro-Psychopharmacology Biol. Psychiatry 87, 56–67. https://doi.org/10.1016/j.pnpbp.2017.09.005

Jentsch, J.D., Taylor, J.R., Redmond, D.E., Elsworth, J.D., Youngren, K.D., Roth, R.H., 1999. Dopamine D4 receptor antagonist reversal of subchronic phencyclidine-induced object

retrieval/detour deficits in monkeys. Psychopharmacology (Berl). 142, 78–84. https://doi.org/10.1007/s002130050865

Johnson, A.R., Thibeault, K.C., Lopez, A.J., Peck, E.G., Sands, L.P., Sanders, C.M., Kutlu, M.G., Calipari, E.S., 2019. Cues play a critical role in estrous cycle-dependent enhancement of cocaine reinforcement. Neuropsychopharmacology 44, 1189–1197. https://doi.org/10.1038/s41386-019-0320-0

Jupp, B., Jones, J.A., Dalley, J.W., 2020. Modelling Differential Vulnerability to Substance Use Disorder in Rodents: Neurobiological Mechanisms. Handb. Exp. Pharmacol. 258, 203–230. https://doi.org/10.1007/164_2019_300

Kalivas, P.W., 2009. The glutamate homeostasis hypothesis of addiction. Nat. Rev. Neurosci. 10, 561–72. https://doi.org/10.1038/nrn2515

Katsouli, S., Stamatakis, A., Giompres, P., Kouvelas, E.D.D., Stylianopoulou, F., Mitsacos, A., 2014. Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain. Neuroscience 257, 49–64. https://doi.org/10.1016/j.neuroscience.2013.10.073

Kelly, L., Farrant, M., Cull-Candy, S.G., 2009. Synaptic mGluR activation drives plasticity of calcium-permeable AMPA receptors. Nat. Neurosci. 12, 593–601. https://doi.org/10.1038/nn.2309

Kerchner, G.A., Nicoll, R.A., 2008. Silent synapses and the emergence of a postsynaptic mechanism for LTP. Nat. Rev. Neurosci. 9, 813–

25. https://doi.org/10.1038/nrn2501

Kikusui, T., Faccidomo, S., Miczek, K.A., 2005. Repeated maternal separation: Differences in cocaine-induced behavioral sensitization in adult male and female mice. Psychopharmacology (Berl). 178, 202–210. https://doi.org/10.1007/s00213-004-1989-1

Kilts, C.D., Schweitzer, J.B., Quinn, C.K., Gross, R.E., Faber, T.L., Muhammad, F., Ely, T.D., Hoffman, J.M., Drexler, K.P.G., 2001. Neural activity related to drug craving in cocaine addiction. Arch. Gen. Psychiatry 58, 334–341. https://doi.org/10.1001/archpsyc.58.4.334

Kleinman, J.E., Sodhi, M.S., Hyde, T.M., Deep-Soboslay, A., Gray, A.L., 2015. Sex differences in glutamate receptor gene expression in major depression and suicide. Mol. Psychiatry 20, 1057–1068. https://doi.org/10.1038/mp.2015.91

Knudsen, E.I., 2004. Sensitive periods in the development of the brain and behavior. J. Cogn. Neurosci. 16, 1412–1425. https://doi.org/10.1162/0898929042304796

Koob, G.F., Buck, C.L., Cohen, A., Edwards, S., Park, P.E., Schlosburg, J.E., Schmeichel, B., Vendruscolo, L.F., Wade, C.L., Whitfield, T.W., George, O., 2014. Addiction as a stress surfeit disorder. Neuropharmacology 76, 370–382. https://doi.org/10.1016/j.neuropharm.2013.05.024

Koob, G.F., Le Moal, M., 2008. Addiction and the Brain Antireward

System. Annu. Rev. Psychol. 59, 29–53. https://doi.org/10.1146/annurev.psych.59.103006.093548

Koob, G.F., Le Moal, M., 2001. Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 24, 97–129. https://doi.org/10.1016/S0893-133X(00)00195-0

Koob, G.F., Zorrilla, E.P., 2010. Neurobiological mechanisms of addiction: focus on corticotropin-releasing factor. Curr. Opin. Investig. Drugs 11, 63–71.

Kosten, T.A., Karanian, D.A., Yeh, J., Haile, C.N., Kim, J.J., Kehoe, P., Bahr, B.A., 2007. Memory impairments and hippocampal modifications in adult rats with neonatal isolation stress experience. Neurobiol. Learn. Mem. 88, 167–176. https://doi.org/10.1016/j.nlm.2007.03.011

Kosten, T.A., Sanchez, H., Zhang, X.Y., Kehoe, P., 2004. Neonatal isolation enhances acquisition of cocaine self-administration and food responding in female rats. Behav. Brain Res. 151, 137–49. https://doi.org/10.1016/j.bbr.2003.08.010

Koya, E., Cruz, F.C., Ator, R., Golden, S. a, Hoffman, A.F., Lupica, C.R., Hope, B.T., 2012. Silent synapses in selectively activated nucleus accumbens neurons following cocaine sensitization. Nat. Neurosci. 15, 1556–1562. https://doi.org/10.1038/nn.3232

Kreibich, A.S., Blendy, J.A., 2004. cAMP Response Element-Binding Protein Is Required for Stress But Not Cocaine-Induced Reinstatement. J. Neurosci. 24, 6686–6692.

https://doi.org/10.1523/JNEUROSCI.1706-04.2004

Kreibich, A.S., Briand, L., Cleck, J.N., Ecke, L., Rice, K.C., Blendy, J.A., 2009. Stress-induced potentiation of cocaine reward: a role for CRF R1 and CREB. Neuropsychopharmacology 34, 2609–17. https://doi.org/10.1038/npp.2009.91

Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894–902. https://doi.org/10.1038/nature07455

Krugers, H.J., Arp, J.M., Xiong, H., Kanatsou, S., Lesuis, S.L., Korosi, A., Joels, M., Lucassen, P.J., 2017. Early life adversity: Lasting consequences for emotional learning. Neurobiol. Stress 6, 14–21. https://doi.org/10.1016/j.ynstr.2016.11.005

Lai, H.M.X., Cleary, M., Sitharthan, T., Hunt, G.E., 2015. Prevalence of comorbid substance use, anxiety and mood disorders in epidemiological surveys, 1990-2014: A systematic review and meta-analysis. Drug Alcohol Depend. https://doi.org/10.1016/j.drugalcdep.2015.05.031

Lane, D.A., Reed, B., Kreek, M.J., Pickel, V.M., 2011. Differential glutamate AMPA-receptor plasticity in subpopulations of VTA neurons in the presence or absence of residual cocaine: Implications for the development of addiction. Neuropharmacology 61, 1129–1140. https://doi.org/10.1016/j.neuropharm.2010.12.031

Larsen, K., Momeni, J., Farajzadeh, L., Callesen, H., Bendixen, C., 2016.

Molecular characterization and analysis of the porcine NURR1 gene. Biochim. Open 3, 26–39. https://doi.org/10.1016/j.biopen.2016.07.001

Larson, E.B., Graham, D.L., Arzaga, R.R., Buzin, N., Webb, J., Green, T.A., Bass, C.E., Neve, R.L., Terwilliger, E.F., Nestler, E.J., Self, D.W., 2011. Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. J. Neurosci. 31, 16447–57. https://doi.org/10.1523/JNEUROSCI.3070-11.2011

Le Moal, M., 2009. Drug abuse: vulnerability and transition to addiction. Pharmacopsychiatry 42 Suppl 1, S42-55. https://doi.org/10.1055/s-0029-1216355

Lee, B.R., Ma, Y.Y., Huang, Y.H., Wang, X., Otaka, M., Ishikawa, M., Neumann, P.A., Graziane, N.M., Brown, T.E., Suska, A., Guo, C., Lobo, M.K., Sesack, S.R., Wolf, M.E., Nestler, E.J., Shaham, Y., Schlüter, O.M., Dong, Y., 2013. Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving. Nat. Neurosci. 16, 1644–1651. https://doi.org/10.1038/nn.3533

Li, C.X., Wang, Y., Gao, H., Pan, W.J., Xiang, Y., Huang, M., Lei, H., 2008. Cerebral metabolic changes in a depression-like rat model of chronic forced swimming studied by ex vivo high resolution 1H magnetic resonance spectroscopy. Neurochem. Res. 33, 2342–2349. https://doi.org/10.1007/s11064-008-9739-0

Lippard, E.T.C., Nemeroff, C.B., 2020. The Devastating Clinical

Consequences of Child Abuse and Neglect: Increased Disease Vulnerability and Poor Treatment Response in Mood Disorders. Am. J. Psychiatry 177, 20–36. https://doi.org/10.1176/appi.ajp.2019.19010020

Lippmann, M., Bress, A., Nemeroff, C.B., Plotsky, P.M., Monteggia, L.M., 2007. Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur. J. Neurosci. 25, 3091–3098. https://doi.org/10.1111/j.1460-9568.2007.05522.x

Lonze, B.E., Ginty, D.D., 2002. Function and Regulation of CREB Family Transcription Factors in the Nervous System CREB and its close relatives are now widely accepted. Neuron 35, 605–623. https://doi.org/10.1016/S0896-6273(02)00828-0

Lowy, M.T., Wittenberg, L., Yamamoto, B.K., 1995. Effect of acute stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. J. Neurochem. 65, 268–74. https://doi.org/10.1046/j.1471-4159.1995.65010268.x

Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10, 434–45. https://doi.org/10.1038/nrn2639

Lüscher, C., 2013a. Cocaine-evoked synaptic plasticity of excitatory transmission in the ventral tegmental area. Cold Spring Harb. Perspect. Med. 3, a012013. https://doi.org/10.1101/cshperspect.a012013

Lüscher, C., 2013b. Drug-Evoked Synaptic Plasticity Causing Addictive Behavior. J. Neurosci. 33, 17641–17646. https://doi.org/10.1523/JNEUROSCI.3406-13.2013

Lynch, W., Roth, M., Carroll, M., 2002. Biological basis of sex differences in drug abuse: preclinical and clinical studies. Psychopharmacology (Berl). 164, 121–137. https://doi.org/10.1007/s00213-002-1183-2

Lynch, W.J., 2008. Acquisition and maintenance of cocaine self-administration in adolescent rats: effects of sex and gonadal hormones. Psychopharmacology (Berl). 197, 237–46. https://doi.org/10.1007/s00213-007-1028-0

Lynch, W.J., Mangini, L.D., Taylor, J.R., 2005. Neonatal isolation stress potentiates cocaine seeking behavior in adult male and female rats. Neuropsychopharmacology 30, 322–9. https://doi.org/10.1038/sj.npp.1300594

Lynch, W.J., Taylor, J.R., 2004. Sex differences in the behavioral effects of 24-h/day access to cocaine under a discrete trial procedure. Neuropsychopharmacology 29, 943–951. https://doi.org/10.1038/sj.npp.1300389

Mahler, S., Smith, R., Aston-Jones, G., 2013. Interactions between VTA orexin and glutamate in cue-induced reinstatement of cocaine seeking in rats. Psychopharmacology (Berl). 226, 687–98. https://doi.org/10.1007/s00213-012-2681-5

Mahler, S. V., Smith, R.J., Aston-Jones, G., 2013. Interactions between

VTA orexin and glutamate in cue-induced reinstatement of cocaine seeking in rats. Psychopharmacology (Berl). 226, 687–698. https://doi.org/10.1007/s00213-012-2681-5

Malenka, R.C., Bear, M.F., 2004. LTP and LTD: an embarrassment of riches. Neuron 44, 5–21. https://doi.org/10.1016/j.neuron.2004.09.012

Mameli, M., Bellone, C., Brown, M.T.C., Lüscher, C., 2011. Cocaine inverts rules for synaptic plasticity of glutamate transmission in the ventral tegmental area. Nat. Neurosci. 14, 414–416. https://doi.org/10.1038/nn.2763

Mangiavacchi, S., Wolf, M.E., 2004. D1 dopamine receptor stimulation increases the rate of AMPA receptor insertion onto the surface of cultured nucleus accumbens neurons through a pathway dependent on protein kinase A. J. Neurochem. 88, 1261–1271. https://doi.org/10.1046/j.1471-4159.2003.02248.x

Marinelli, M., White, F.J., 2000. Enhanced vulnerability to cocaine self-administration is associated with elevated impulse activity of midbrain dopamine neurons. J. Neurosci. 20, 8876–8885. https://doi.org/20/23/8876 [pii]

Martínez-Rivera, A., Hao, J., Tropea, T.F., Giordano, T.P., Kosovsky, M., Rice, R.C., Lee, A., Huganir, R.L., Striessnig, J., Addy, N.A., Han, S., Rajadhyaksha, A.M., 2017. Enhancing VTA Ca v 1.3 L-type Ca2+channel activity promotes cocaine and mood-related behaviors via overlapping AMPA receptor mechanisms in the nucleus accumbens. Mol. Psychiatry 22, 1735–1745.

https://doi.org/10.1038/mp.2017.9

Matthews, K., Robbins, T.W., 2003. Early experience as a determinant of adult behavioural responses to reward: the effects of repeated maternal separation in the rat. Neurosci. Biobehav. Rev. 27, 45–55. https://doi.org/10.1016/s0149-7634(03)00008-3

Mattson, B.J., Bossert, J.M., Simmons, D.E., Nozaki, N., Nagarkar, D., Kreuter, J.D., Hope, B.T., 2005. Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats is enabled by enhanced activation of extracellular signal-related kinase, but not protein kinase A. J. Neurochem. 95, 1481–1494. https://doi.org/10.1111/j.1471-4159.2005.03500.x

McClung, C.A., Nestler, E.J., 2003. Regulation of gene expression and cocaine reward by CREB and FosB. Nat. Neurosci. 6, 1208–1215. https://doi.org/10.1038/nn1143

McFarland, K., Lapish, C.C., Kalivas, P.W., 2003. Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. J. Neurosci. 23, 3531–3537. https://doi.org/10.1523/jneurosci.23-08-03531.2003

McHugh, C.M., Chun Lee, R.S., Hermens, D.F., Corderoy, A., Large, M., Hickie, I.B., 2019. Impulsivity in the self-harm and suicidal behavior of young people: A systematic review and meta-analysis. J. Psychiatr. Res. 116, 51–60. https://doi.org/10.1016/j.jpsychires.2019.05.012

McHugh, R.K., Votaw, V.R., Sugarman, D.E., Greenfield, S.F., 2018.

Sex and gender differences in substance use disorders. Clin. Psychol. Rev. 66, 12–23. https://doi.org/10.1016/j.cpr.2017.10.012

McLaughlin, J.P., Marton-Popovici, M., Chavkin, C., 2003. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J. Neurosci. 23, 5674–83.

Ménard, C., Hodes, G.E., Russo, S.J., 2016. Pathogenesis of depression: Insights from human and rodent studies. Neuroscience 321, 138–162. https://doi.org/10.1016/j.neuroscience.2015.05.053

Merz, K., Herold, S., Lie, D.C., 2011. CREB in adult neurogenesis - master and partner in the development of adult-born neurons? Eur. J. Neurosci. 33, 1078–1086. https://doi.org/10.1111/j.1460-9568.2011.07606.x

Miczek, K.A., Yap, J.J., Covington, H.E., 2008. Social stress, therapeutics and drug abuse: Preclinical models of escalated and depressed intake. Pharmacol. Ther. 120, 102–128. https://doi.org/10.1016/J.PHARMTHERA.2008.07.006

Moffett, M., Harley, J., Francis, D., Sanghani, S.P., Davis, W.I., Kuhar, M.J., 2006. Maternal separation and handling affects cocaine self-administration in both the treated pups as adults and the dams. J. Pharmacol. Exp. Ther. 317, 1210–1218. https://doi.org/10.1124/jpet.106.101139

Morein-Zamir, S., Simon Jones, P., Bullmore, E.T., Robbins, T.W.,

Ersche, K.D., 2015. Take it or leave it: Prefrontal control in recreational cocaine users. Transl. Psychiatry 5, e582–e582. https://doi.org/10.1038/tp.2015.80

Mulder, C.L., Serrano, J.B., Catsburg, L.A.E., Roseboom, T.J., Repping, S., Van Pelt, A.M.M., 2018. A practical blueprint to systematically study life-long health consequences of novel medically assisted reproductive treatments. Hum. Reprod. 33, 784–792. https://doi.org/10.1093/humrep/dey070

Muschamp, J.W., Carlezon, W.A., 2013. Roles of nucleus accumbens CREB and dynorphin in dysregulation of motivation. Cold Spring Harb. Perspect. Med. 3, a012005–a012005. https://doi.org/10.1101/cshperspect.a012005

Nakamura, K., Kurasawa, M., Shirane, M., 2000. Impulsivity and AMPA receptors: aniracetam ameliorates impulsive behavior induced by a blockade of AMPA receptors in rats. Brain Res. 862, 266–9. https://doi.org/10.1016/s0006-8993(00)02160-0

Narayanan, N.S., Laubach, M., 2017. Inhibitory Control: Mapping Medial Frontal Cortex. Curr. Biol. 27, R148–R150. https://doi.org/10.1016/j.cub.2017.01.010

Nelson, C.A., Gabard-Durnam, L.J., 2020. Early Adversity and Critical Periods: Neurodevelopmental Consequences of Violating the Expectable Environment. Trends Neurosci. 43, 133–143. https://doi.org/10.1016/j.tins.2020.01.002

Nestler, E.J., 2015. Role of the Brain's Reward Circuitry in Depression:

Transcriptional Mechanisms. Int. Rev. Neurobiol. 124, 151–70. https://doi.org/10.1016/bs.irn.2015.07.003

Nestler, E.J., 2005. Is there a common molecular pathway for addiction? Nat. Neurosci. 8, 1445–9. https://doi.org/10.1038/nn1578

Nestler, E.J., 2004. Historical review: Molecular and cellular mechanisms of opiate and cocaine addiction. Trends Pharmacol. Sci. 25, 210–218. https://doi.org/10.1016/J.TIPS.2004.02.005

Nestler, E.J., 2001. Molecular basis of long-term plasticity underlying addiction. Nat. Rev. Neurosci. 2, 119–28. https://doi.org/10.1038/35053570

Nestler, E.J., Barrot, M., DiLeone, R.J., Eisch, A.J., Gold, S.J., Monteggia, L.M., 2002. Neurobiology of depression. Neuron 34, 13–25. https://doi.org/10.1016/s0896-6273(02)00653-0

Nestler, E.J., Carlezon, W.A., 2006. The Mesolimbic Dopamine Reward Circuit in Depression. Biol. Psychiatry 59, 1151–1159. https://doi.org/10.1016/J.BIOPSYCH.2005.09.018

Newman, E.L., Leonard, M.Z., Arena, D.T., de Almeida, R.M.M., Miczek, K.A., 2018. Social defeat stress and escalation of cocaine and alcohol consumption: Focus on CRF. Neurobiol. Stress 9, 151–165. https://doi.org/10.1016/j.ynstr.2018.09.007

Nicholls, J., Staiger, P.K., Williams, J.S., Richardson, B., Kambouropoulos, N., 2014. When social anxiety co-occurs with substance use: Does an impulsive social anxiety subtype explain this unexpected relationship? Psychiatry Res. 220, 909–914.

https://doi.org/10.1016/j.psychres.2014.08.040

Olson, V.G., Zabetian, C.P., Bolanos, C.A., Edwards, S., Barrot, M., Eisch, A.J., Hughes, T., Self, D.W., Neve, R.L., Nestler, E.J., 2005. Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. J. Neurosci. 25, 5553–62. https://doi.org/10.1523/JNEUROSCI.0345-05.2005

Orso, R., Creutzberg, K.C., Wearick-Silva, L.E., Wendt Viola, T., Tractenberg, S.G., Benetti, F., Grassi-Oliveira, R., 2019. How Early Life Stress Impact Maternal Care: A Systematic Review of Rodent Studies. Front. Behav. Neurosci. 13. https://doi.org/10.3389/fnbeh.2019.00197

Park, W.-K., Bari, A.A., Jey, A.R., Anderson, S.M., Spealman, R.D., Rowlett, J.K., Pierce, R.C., 2002. Cocaine administered into the medial prefrontal cortex reinstates cocaine-seeking behavior by increasing AMPA receptor-mediated glutamate transmission in the nucleus accumbens. J. Neurosci. 22, 2916–25. https://doi.org/20026235

Park, W.K., Bari, A.A., Jey, A.R., Anderson, S.M., Spealman, R.D., Rowlett, J.K., Pierce, R.C., 2002. Cocaine Administered into the Medial Prefrontal Cortex Reinstates Cocaine-Seeking Behavior by Increasing AMPA Receptor-Mediated Glutamate Transmission in the Nucleus Accumbens. J. Neurosci. 22, 2916–2925. https://doi.org/10.1523/jneurosci.22-07-02916.2002

Patel, V., Chisholm, D., Parikh, R., Charlson, F.J., Degenhardt, L., Dua,

T., Ferrari, A.J., Hyman, S., Laxminarayan, R., Levin, C., Lund, C., Medina Mora, M.E., Petersen, I., Scott, J., Shidhaye, R., Vijayakumar, L., Thornicroft, G., Whiteford, H., DCP MNS Author Group, 2016. Addressing the burden of mental, neurological, and substance use disorders: key messages from Disease Control Priorities, 3rd edition. Lancet (London, England) 387, 1672–85. https://doi.org/10.1016/S0140-6736(15)00390-6

Peluso, M.A.M., Hatch, J.P., Glahn, D.C., Monkul, E.S., Sanches, M., Najt, P., Bowden, C.L., Barratt, E.S., Soares, J.C., 2007. Trait impulsivity in patients with mood disorders. J. Affect. Disord. 100, 227–231. https://doi.org/10.1016/j.jad.2006.09.037

Penberthy, J.K., Ait-Daoud, N., Vaughan, M., Fanning, T., 2010. Review of treatment for cocaine dependence. Curr. Drug Abuse Rev. 3, 49–62. https://doi.org/10.2174/1874473711003010049

Perry, J.L., Larson, E.B., German, J.P., Madden, G.J., Carroll, M.E., 2005. Impulsivity (delay discounting) as a predictor of acquisition of IV cocaine self-administration in female rats. Psychopharmacology (Berl). 178, 193–201. https://doi.org/10.1007/s00213-004-1994-4

Perry, J.L., Nelson, S.E., Carroll, M.E., 2008. Impulsive Choice As a Predictor of Acquisition of IV Cocaine Self-Administration and Reinstatement of Cocaine-Seeking Behavior in Male and Female Rats. Exp. Clin. Psychopharmacol. 16, 165–177. https://doi.org/10.1037/1064-1297.16.2.165

Peterson, A.B., Hivick, D.P., Lynch, W.J., 2014. Dose-dependent

effectiveness of wheel running to attenuate cocaine-seeking: Impact of sex and estrous cycle in rats. Psychopharmacology (Berl). 231, 2661–2670. https://doi.org/10.1007/s00213-014-3437-1

Pickering, C., Gustafsson, L., Cebere, A., Nylander, I., Liljequist, S., 2006. Repeated maternal separation of male Wistar rats alters glutamate receptor expression in the hippocampus but not the prefrontal cortex. Brain Res. 1099, 101–108. https://doi.org/10.1016/j.brainres.2006.04.136

Pierce, R.C., Wolf, M.E., 2013. Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. Cold Spring Harb. Perspect. Med. 3, a012021. https://doi.org/10.1101/cshperspect.a012021

Ping, A., Xi, J., Prasad, B.M., Wang, M.H., Kruzich, P.J., 2008. Contributions of nucleus accumbens core and shell GluR1 containing AMPA receptors in AMPA- and cocaine-primed reinstatement of cocaine-seeking behavior. Brain Res. 1215, 173–182. https://doi.org/10.1016/j.brainres.2008.03.088

Pirot, S., Godbout, R., Mantz, J., Tassin, J.P., Glowinski, J., Thierry, A.M., 1992. Inhibitory effects of ventral tegmental area stimulation on the activity of prefrontal cortical neurons: evidence for the involvement of both dopaminergic and GABAergic components. Neuroscience 49, 857–65. https://doi.org/10.1016/0306-4522(92)90362-6

Planchez, B., Surget, A., Belzung, C., 2019. Animal models of major

depression: drawbacks and challenges. J. Neural Transm. 126, 1383–1408. https://doi.org/10.1007/s00702-019-02084-y

Portero-Tresserra, M., Gracia-Rubio, I., Cantacorps, L., Pozo, O.J., Gómez-Gómez, A., Pastor, A., López-Arnau, R., de la Torre, R., Valverde, O., 2018. Maternal separation increases alcohol-drinking behaviour and reduces endocannabinoid levels in the mouse striatum and prefrontal cortex. Eur. Neuropsychopharmacol. 28, 499–512. https://doi.org/10.1016/j.euroneuro.2018.02.003

Purves, D., Augustine, G., Fitzpatrick, D., Hall, W., Anthony-Samuel, L., McNamara, J., Williams, M., 2004. Neuroscience, 3rd ed. ed, Sinauer Associates, Inc.

Rappeneau, V., Bérod, A., 2017. Reconsidering depression as a risk factor for substance use disorder: Insights from rodent models. Neurosci. Biobehav. Rev. 77, 303–316. https://doi.org/10.1016/j.neubiorev.2017.04.001

Récamier-Carballo, S., Estrada-Camarena, E., López-Rubalcava, C., 2017. Maternal separation induces long-term effects on monoamines and brain-derived neurotrophic factor levels on the frontal cortex, amygdala, and hippocampus: differential effects after a stress challenge. Behav. Pharmacol. 28, 545–557. https://doi.org/10.1097/FBP.0000000000000324

Riday, T., Kosofsky, B., Malanga, C., 2012. The rewarding and locomotor-sensitizing effects of repeated cocaine administration are distinct and separable in mice. Neuropharmacology 62, 1858–66. https://doi.org/10.1016/j.neuropharm.2011.12.011

Río-Álamos, C., Piludu, M.A., Gerbolés, C., Barroso, D., Oliveras, I., Sánchez-González, A., Cañete, T., Tapias-Espinosa, C., Sampedro-Viana, D., Torrubia, R., Tobeña, A., Fernández-Teruel, A., 2019. Volumetric brain differences between the Roman rat strains: Neonatal handling effects, sensorimotor gating and working memory. Behav. Brain Res. 361, 74–85. https://doi.org/10.1016/j.bbr.2018.12.033

Ritchie, H., Roser, M., 2018. Opioids, cocaine, cannabis and illicit drugs [WWW Document]. URL https://ourworldindata.org/illicit-drug-use#citation (accessed 4.13.20).

Roberto, M., Gilpin, N.W., 2014. Central Amygdala Neuroplasticity in Alcohol Dependence, in: Neurobiology of Alcohol Dependence. Elsevier, pp. 207–226. https://doi.org/10.1016/B978-0-12-405941-2.00011-0

Romano-López, A., Méndez-Díaz, M., García García, F., Regalado-Santiago, C., Ruiz-Contreras, A.E., Prospéro-García, O., García, F.G., Regalado-Santiago, C., Ruiz-Contreras, A.E., Prospéro-García, O., 2015. Maternal separation and early stress cause long-lasting effects on dopaminergic and endocannabinergic systems and alters dendritic morphology in the nucleus accumbens and frontal cortex in rats. Dev. Neurobiol. 76, 819–831. https://doi.org/10.1002/dneu.22361

Rømer Thomsen, K., Callesen, M.B., Hesse, M., Kvamme, T.L., Pedersen, M.M., Pedersen, M.U., Voon, V., 2018. Impulsivity traits and addiction-related behaviors in youth. J. Behav. Addict. 7, 317–

330. https://doi.org/10.1556/2006.7.2018.22

Russo, S., Nestler, E., 2013. The brain reward circuitry in mood disorders. Nat. Rev. Neurosci. 625, 609–625. https://doi.org/10.1038/nrn3381

Russo, S.J., Dietz, D.M., Dumitriu, D., Morrison, J.H., Malenka, R.C., Nestler, E.J., 2010. The addicted synapse: Mechanisms of synaptic and structural plasticity in nucleus accumbens. Trends Neurosci. 33, 267–276. https://doi.org/10.1016/j.tins.2010.02.002

Sachs, B.D., Tran, H.L., Folse, E., Caron, M.G., 2018. Brain-region-specific Molecular Responses to Maternal Separation and Social Defeat Stress in Mice. Neuroscience 373, 122–136. https://doi.org/10.1016/j.neuroscience.2018.01.018

Salamone, J.D., Correa, M., 2012. The Mysterious Motivational Functions of Mesolimbic Dopamine. Neuron 76, 470–485. https://doi.org/10.1016/j.neuron.2012.10.021

Sanvicente-Vieira, B., Rovaris, D.L., Ornell, F., Sordi, A., Rothmann, L.M., Niederauer, J.P.O., Schuch, J.B., von Diemen, L., Kessler, F.H.P., Grassi-Oliveira, R., 2019. Sex-based differences in multidimensional clinical assessments of early-abstinence crack cocaine users. PLoS One 14. https://doi.org/10.1371/journal.pone.0218334

Schenk, S., Snow, S., 1994. Sensitization to cocaine's motor activating properties produced by electrical kindling of the medial prefrontal cortex but not of the hippocampus. Brain Res. 659, 17–22.

https://doi.org/10.1016/0006-8993(94)90858-3

Schmidt, M. V., 2010. Molecular mechanisms of early life stress--lessons from mouse models. Neurosci. Biobehav. Rev. 34, 845–52. https://doi.org/10.1016/j.neubiorev.2009.05.002

Shu, C., Xiao, L., Tang, J., Wang, G., Zhang, X., Wang, X., 2015. Blunted behavioral and molecular responses to chronic mild stress in adult rats with experience of infancy maternal separation. Tohoku J. Exp. Med. 235, 81–7. https://doi.org/10.1620/tjem.235.81

Shutoh, F., Hamada, S., Shibata, M., Narita, M., Shiga, T., Azmitia, E.C., Okado, N., 2000. Long term depletion of serotonin leads to selective changes in glutamate receptor subunits. Neurosci. Res. 38, 365–371. https://doi.org/10.1016/S0168-0102(00)00184-X

Skolnick, P., 2008. AMPA receptors: a target for novel antidepressants? Biol. Psychiatry 63, 347–8. https://doi.org/10.1016/j.biopsych.2007.10.011

Sondheimer, I., Knackstedt, L.A., 2011. Ceftriaxone prevents the induction of cocaine sensitization and produces enduring attenuation of cue- and cocaine-primed reinstatement of cocaine-seeking. Behav. Brain Res. 225, 252–258. https://doi.org/10.1016/j.bbr.2011.07.041

Squire, L.R., 2008. Fundamental Neuroscience, 3rd ed. ed. https://doi.org/10.1097/00005072-199712000-00013

Stuber, G.D., Hopf, F.W., Tye, K.M., Chen, B.T., Bonci, A., 2010.

Neuroplastic alterations in the limbic system following cocaine or alcohol exposure. Curr. Top. Behav. Neurosci. 3, 3–27. https://doi.org/10.1007/7854_2009_23

Suderman, M., McGowan, P.O., Sasaki, A., Huang, T.C.T., Hallett, M.T., Meaney, M.J., Turecki, G., Szyf, M., 2012. Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus. Proc. Natl. Acad. Sci. U. S. A. 109, 17266–17272. https://doi.org/10.1073/pnas.1121260109

Sun, X., Zhao, Y., Wolf, M.E., 2005. Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. J. Neurosci. 25, 7342–7351. https://doi.org/10.1523/JNEUROSCI.4603-04.2005

Suto, N., Ecke, L.E., Wise, R.A., 2009a. Control of within-binge cocaine-seeking by dopamine and glutamate in the core of nucleus accumbens. Psychopharmacology (Berl). 205, 431–439. https://doi.org/10.1007/s00213-009-1553-0

Suto, N., Ecke, L.E., Wise, R.A., 2009b. Control of within-binge cocaine-seeking by dopamine and glutamate in the core of nucleus accumbens. Psychopharmacology (Berl). 205, 431–439. https://doi.org/10.1007/s00213-009-1553-0

Svenningsson, P., Tzavara, E.T., Witkin, J.M., Fienberg, A.A., Nomikos, G.G., Greengard, P., 2002. Involvement of striatal and extrastriatal DARPP-32 in biochemical and behavioral effects of fluoxetine (Prozac). Proc. Natl. Acad. Sci. U. S. A. 99, 3182–3187. https://doi.org/10.1073/pnas.052712799

Swalve, N., Smethells, J.R., Carroll, M.E., 2016. Sex differences in the acquisition and maintenance of cocaine and nicotine self-administration in rats. Psychopharmacology (Berl). 233, 1005–13. https://doi.org/10.1007/s00213-015-4183-8

Swanson, L.W., 2013. Brain Architecture [WWW Document]. Brain Archit. https://doi.org/10.1093/med/9780195378580.001.0001

Swendsen, J., Le Moal, M., 2011. Individual vulnerability to addiction. Ann. N. Y. Acad. Sci. 1216, 73–85. https://doi.org/10.1111/j.1749-6632.2010.05894.x

Sylva, K., 1997. Critical periods in childhood learning. Br. Med. Bull. 53, 185–97. https://doi.org/10.1093/oxfordjournals.bmb.a011599

Tan, C.H., He, X., Yang, J., Ong, W.Y., 2006. Changes in AMPA subunit expression in the mouse brain after chronic treatment with the antidepressant maprotiline: A link between noradrenergic and glutamatergic function? Exp. Brain Res. 170, 448–456. https://doi.org/10.1007/s00221-005-0228-2

Tang, W.-X., Fasulo, W., Mash, D., Hemby, S., 2003. Molecular profiling of midbrain dopamine regions in cocaine overdose victims. J. Neurochem. 85, 911–24. https://doi.org/10.1046/j.1471-4159.2003.01740.x

Tang, W., Wesley, M., Freeman, W., Liang, B., Hemby, S., 2004. Alterations in ionotropic glutamate receptor subunits during binge cocaine self-administration and withdrawal in rats. J. Neurochem. 89, 1021–33. https://doi.org/10.1111/j.1471-4159.2004.02392.x

Tarantola, D., 2018. Child Maltreatment: Daunting and Universally Prevalent. Am. J. Public Health 108, 1119–1120. https://doi.org/10.2105/AJPH.2018.304637

Teicher, M.H., Samson, J.A., 2016. Annual Research Review: Enduring neurobiological effects of childhood abuse and neglect. J. Child Psychol. Psychiatry. 57, 241–66. https://doi.org/10.1111/jcpp.12507

Teicher, M.H., Samson, J.A., Anderson, C.M., Ohashi, K., 2016. The effects of childhood maltreatment on brain structure, function and connectivity. Nat. Rev. Neurosci. 17, 652–66. https://doi.org/10.1038/nrn.2016.111

The jackson laboratory, 2017. Life span as a biomarker [WWW Document]. URL https://www.jax.org/research-and-faculty/research-labs/the-harrison-lab/gerontology/life-span-as-a-biomarker# (accessed 3.26.20).

Torrens, M., Mestre-Pintó, J.-I., Domingo-Salvany, A., Torrens, Marta, Mestre-Pintó, Joan-Ignasi and Domingo-Salvany, A., 2015. Comorbidity of substance use and mental disorders in Europe, European Monitioring Centre for Drugs and Drug Addiction. Publications Office. https://doi.org/10.2810/532790

Tractenberg, S.G., Levandowski, M.L., de Azeredo, L.A., Orso, R., Roithmann, L.G., Hoffmann, E.S., Brenhouse, H., Grassi-Oliveira, R., 2016. An overview of maternal separation effects on behavioural outcomes in mice: Evidence from a four-stage methodological systematic review. Neurosci. Biobehav. Rev. 68,

489–503. https://doi.org/10.1016/j.neubiorev.2016.06.021

Turner, B.D., Kashima, D.T., Manz, K.M., Grueter, C.A., Grueter, B.A., 2018. Synaptic Plasticity in the Nucleus Accumbens: Lessons Learned from Experience. Neural Mech. Addict. 9, 2114–2126. https://doi.org/10.1021/acschemneuro.7b00420

UN General Assembly, 1989. Convention on the rights of the child, Treaty Series.

UNODC, 2019. World Drug Report 2019.

UNODC, 2018. World Drug Report 2018.

UNODC, 2016. World drug report 2016.

Uutela, A., 2001. Drugs: Illicit Use and Prevention, in: International Encyclopedia of the Social & Behavioral Sciences. Pergamon, pp. 3877–3881. https://doi.org/10.1016/b0-08-043076-7/03886-9

Valvassori, S.S., Varela, R.B., Quevedo, J., 2017. Animal Models of Mood Disorders: Focus on Bipolar Disorder and Depression, in: Animal Models for the Study of Human Disease: Second Edition. Elsevier Inc., pp. 991–1001. https://doi.org/10.1016/B978-0-12-809468-6.00038-3

Van Haaren, F., Meyer, M.E., 1991. Sex differences in locomotor activity after acute and chronic cocaine administration. Pharmacol. Biochem. Behav. 39, 923–927. https://doi.org/10.1016/0091-3057(91)90054-6

van Harmelen, A.-L., van Tol, M.-J., Demenescu, L.R., van der Wee,

N.J.A., Veltman, D.J., Aleman, A., van Buchem, M.A., Spinhoven, P., Penninx, B.W.J.H., Elzinga, B.M., 2013. Enhanced amygdala reactivity to emotional faces in adults reporting childhood emotional maltreatment. Soc. Cogn. Affect. Neurosci. 8, 362–9. https://doi.org/10.1093/scan/nss007

Vetulani, J., 2013. Early maternal separation: a rodent model of depression and a prevailing human condition. Pharmacol. Rep. 65, 1451–61. https://doi.org/10.1016/S1734-1140(13)71505-6

Vialou, V., Robison, A.J., Laplant, Q.C., Covington Iii, H.E., Dietz, D.M., Ohnishi, Y.N., Mouzon, E., Rush Iii, A.J., Watts, E.L., Wallace, D.L., Iniguez, S.D., Ohnishi, Y.H., Steiner, M.A., Warren, B., Krishnan, V., Neve, R.L., Ghose, S., Berton, O., Tamminga, C.A., Nestler, E.J., 2010. ΔFosB in brain reward circuits mediates resilience to stress and antidepressant responses. Nat Neurosci 13, 745–752. https://doi.org/10.1038/nn.2551

Viola, T.W., Wearick-Silva, L.E., De Azeredo, L.A.A., Centeno-Silva, A., Murphy, C., Marshall, P., Li, X., Singewald, N., Garcia, F., Bredy, T.W., Grassi-Oliveira, R., 2016. Increased cocaine-induced conditioned place preference during periadolescence in maternally separated male BALB/c mice: the role of cortical BDNF, microRNA-212, and MeCP2. Psychopharmacology (Berl). 233, 3279–3288. https://doi.org/10.1007/s00213-016-4373-z

Volkow, N.D., Koob, G.F., McLellan, A.T., 2016. Neurobiologic Advances from the Brain Disease Model of Addiction. N. Engl. J. Med. 374, 363–71. https://doi.org/10.1056/NEJMra1511480

Volkow, N.D., Michaelides, M., Baler, R., 2019. The Neuroscience of Drug Reward and Addic-tion. Physiol Rev 99, 2115–2140. https://doi.org/10.1152/physrev.00014.2018

Wagner, F.A., Anthony, J.C., 2002. From first drug use to drug dependence; developmental periods of risk for dependence upon marijuana, cocaine, and alcohol. Neuropsychopharmacology 26, 479–88. https://doi.org/10.1016/S0893-133X(01)00367-0

Wang, Y.Y., Jiang, N.Z., Cheung, E.F.C., Sun, H.W., Chan, R.C.K., 2015. Role of depression severity and impulsivity in the relationship between hopelessness and suicidal ideation in patients with major depressive disorder. J. Affect. Disord. 183, 83–89. https://doi.org/10.1016/j.jad.2015.05.001

Weafer, J., de Wit, H., 2014. Sex differences in impulsive action and impulsive choice. Addict. Behav. 39, 1573–1579. https://doi.org/10.1016/j.addbeh.2013.10.033

WHO, 2017. Depression and Other Common Mental Disorders: Global Health Estimates.

WHO, 2016. INSPIRE: Seven strategies for ending violence against children. World Heal. Organ. 108.

WHO, 2006. Preventing child maltreatment: a guide.

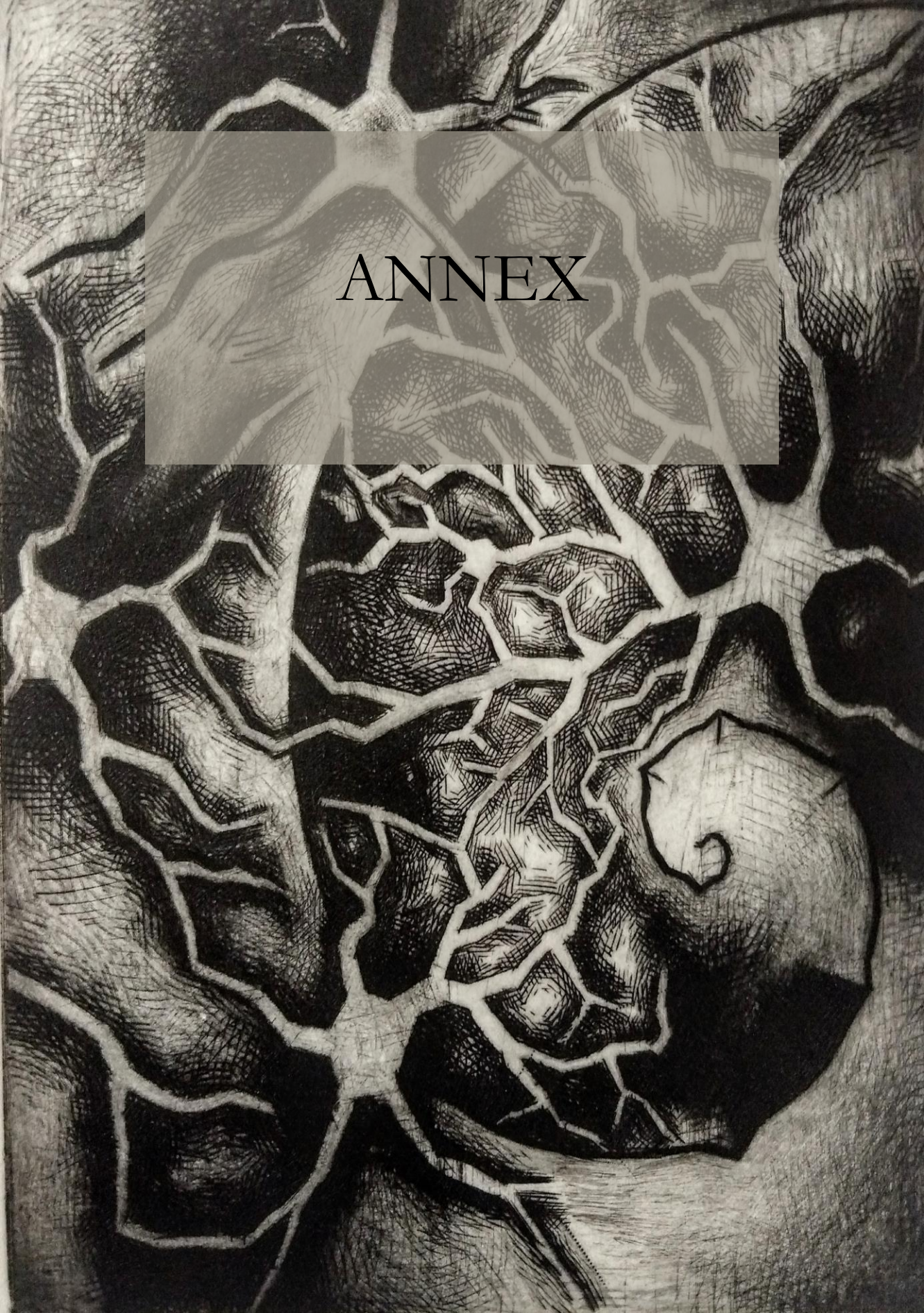
Wickens, M.M., Deutschmann, A.U., McGrath, A.G., Parikh, V., Briand, L.A., 2019. Glutamate receptor interacting protein acts within the prefrontal cortex to blunt cocaine seeking. Neuropharmacology 157, 107672.

https://doi.org/10.1016/j.neuropharm.2019.107672

Yang, Y., Wang, X.-B., Zhou, Q., 2010. Perisynaptic GluR2-lacking AMPA receptors control the reversibility of synaptic and spines modifications. Proc. Natl. Acad. Sci. U. S. A. 107, 11999–2004. https://doi.org/10.1073/pnas.0913004107

Yang, Y., Wang, X. -b., Frerking, M., Zhou, Q., 2008. Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long-term potentiation. Proc. Natl. Acad. Sci. 105, 11388–11393. https://doi.org/10.1073/pnas.0802978105

Zlebnik, N.E., 2019. Females pay a higher price for addiction. Neuropsychopharmacology 44, 1179–1181. https://doi.org/10.1038/s41386-019-0373-0



ANNEX

Annex 1

Repeated Cannabidiol treatment reduces cocaine intake and modulates neural proliferation and CB1R expression in the mouse hippocampus.

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Neuropharmacology 143: 163-175 (2018)

DOI: 10.1016/j.neuropharm.2018.09.043

Neuropharmacology 143 (2018) 163–175

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Repeated Cannabidiol treatment reduces cocaine intake and modulates neural proliferation and CB1R expression in the mouse hippocampus

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HIGHLIGHTS

- CBD treatment induces anxiolytic and cognitive effects showing a bell-shaped dose-response curve.
- CBD treatment reduces cocaine-induced conditioned place preference, but not behavioural sensitization.
- CBD attenuates cocaine intake and breaking point but does not alter reinstatement of cocaine-seeking behaviour.
- CBD increases CB1R expression and neural progenitor proliferation in the hippocampus of cocaine self-administering animals.

ARTICLE INFO

Keywords:
Cocaine
Cannabidiol
Self-administration
Addiction
Neurogenesis

ABSTRACT

Cannabinoid derivatives have shown promising results for treating neuropsychiatric disorders, including drug addiction. Recent studies on the therapeutic effects of Cannabidiol (CBD) on drug abuse showed mixed results, especially with psychostimulant substances such as cocaine. To determine whether CBD can attenuate cocaine reinforcement, we assessed behavioural responses induced by cocaine in mice, using the behavioural sensitization, conditioned place preference and intravenous self-administration paradigms. We show that repeated CBD treatment produces anxiolytic effects in the elevated plus maze test, increases the discrimination index of the novel object recognition task and attenuates cocaine-induced conditioned place preference but does not affect behavioural sensitization. CBD reduced cocaine voluntary consumption and progressive ratio breaking point in the self-administration paradigm, but not drug-induced reinstatement. In parallel, CBD increased expression of type 1 cannabinoid receptor, MAPK-CREB phosphorylation, BDNF expression, and neural cell proliferation in the hippocampus, and reduced the GluA1/2 AMPA subunit receptor ratio in the striatum. In summary, we show that CBD can modulate some behavioural and molecular manifestations of cocaine reinforcement. Moreover, our findings show that CBD has pro-neurogenic effects also in cocaine consuming animals. Overall, this novel evidence provides new perspectives to use CBD as a therapeutic tool.

1. Introduction

Cocaine addiction is a chronic and relapsing disease characterized by compulsive drug seeking and use, despite its harmful consequences (Volkow et al., 2016). Repeated cocaine use promotes neural plasticity processes that cause aberrant motivation towards the drug and related stimuli, which can produce neurobiological alterations leading to drug addiction (Everitt et al., 2018; Pascoli et al., 2015). Epidemiological studies show that cocaine is the second most widely consumed illicit drug in Europe (EMCDDA, 2016) and the United States (CBHSQ, 2015). There are no effective treatments, however, so it is required to develop innovative therapeutic strategies (Czoty et al., 2016).

Cannabinoids, as cannabidiol (CBD), have drawn interest from clinical and preclinical researchers as a strategy for treating substance use disorders (Ware, 2018; Wenzel and Cheer, 2018). CBD is the most abundant, non-psychoactive cannabinoid among the more than eighty

Abbreviations: 5-HT$_{1A}$R, 5-hydroxytryptamine 1 A receptor; ANOVA, Analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; CB1R, Type 1 cannabinoid receptor; CB2R, Type 2 cannabinoid receptor; CBD, Cannabidiol; CREB, cAMP response element-binding protein; ERK1/2, Extracellular signal–regulated kinases 1/2; GluA1/2, AMPA receptor subunit 1/2; MAPK, Mitogen-Activated Protein Kinases; NeuN, Neuronal Nuclei; THC, Δ^9-tetrahydrocannabinol

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https://doi.org/10.1016/j.neuropharm.2018.09.043
Received 28 June 2018; Received in revised form 4 September 2018; Accepted 26 September 2018
Available online 28 September 2018
0028-3908/

compounds present in *Cannabis sativa* plant. CBD has a multi-target pharmacological profile, it can act as a 5-hydroxytryptamine 1 A receptor (5-HT$_{1A}$R) agonist (Russo et al., 2005), a μ-opioid receptor positive allosteric modulator (Kathmann et al., 2006), an adenosine uptake inhibitor (Liou et al., 2008), a negative allosteric modulator of type 1 (CB1R) (Laprairie et al., 2015) and type 2 cannabinoid receptors (CB2R) (Martínez-Pinilla et al., 2017), and an inhibitor of fatty acid amide hydrolase activity (Chauvet et al., 2015). So far, CBD has been outlined as an anxiolytic agent (Campos et al., 2013) with antipsychotic (Renard et al., 2016) and antidepressant (Schiavon et al., 2016) properties. CBD can also attenuate cue-induced heroin-seeking in rats (Ren et al., 2009) and humans (Hurd et al., 2015), reduces morphine-induced conditioned place preference (Markos et al., 2017), and facilitates intracranial self-stimulation in rats (Katsidoni et al., 2013). Research on CBD and psychostimulants-induced behavioural effects have revealed contradictory results. CBD disrupts extinction of conditioned place preference induced by cocaine and amphetamine (Parker et al., 2004), impairs reconsolidation of contextual morphine-associated memories in the same paradigm (de Carvalho and Takahashi, 2017), and blocks amphetamine-induced behavioural sensitization in rats (Renard et al., 2016). In contrast, CBD does not inhibit the reward-facilitating effects of cocaine on intracranial self-stimulation (Katsidoni et al., 2013), and does not modify context-specific reversal of cocaine sensitization (Gerdeman et al., 2008). Recently, Mahmud et al. (2017) showed that acute CBD administration does not influence cocaine self-administration in rats. However, a 7-day treatment based on transdermal delivery of CBD robustly attenuated cue-induced reinstatement of cocaine self-administration in rats (Gonzalez-Cuevas et al., 2018).

These discrepancies in the literature evidence that the pharmacological effects and targets of CBD and its protective actions on drug addiction are still poorly understood. So far, there are different neurobiological mechanisms that could support a putative effect of CBD over the motivational dysfunctions associated with cocaine intake. Recent studies proved the modulation of dopamine release in the nucleus accumbens (Nac) by CBD (Renard et al., 2016) through a mechanism involving the activation of 5-HT$_{1A}$ receptors (Norris et al., 2016). As a result, CBD-treated animals showed a robust reduction in the amphetamine-induced locomotor sensitization. Moreover, evidences confirm the hippocampal pro-neurogenic effects of CBD (Campos et al., 2017). CBD induced cell proliferation and hippocampal neurogenesis after repeated treatment in mice (Schiavon et al., 2016). Interestingly, the increased rate of adult hippocampal neurogenesis induced by CBD was necessary to observe some of its behavioural-related changes. For instance, Campos et al. (2013) reported that the anxiolytic effect of CBD on chronically stressed mice depended on hippocampal neurogenesis. Increased activity-dependent levels of neural progenitor proliferation in the dentate gyrus have been consistently linked to a reduction of drug taking and increased neuroprotection (for an extensive review see Chambers, 2013). More specifically, Wolf et al. (2010) and Campos et al. (2013) have suggested an involvement of CB1R transmission in this possible mechanism of CBD. In this sense, mitogen-activated protein kinases/cAMP response element-binding protein (MAPK/CREB) signalling pathway has been described as a plausible link between increased CB1R transmission and neural proliferation enhancement (Prenderville et al., 2015). Overall, preclinical published results led us to hypothesize that: (1) the reduction of neural proliferation commonly observed after cocaine consumption could be attenuated in CBD-treated animals (Castilla-Ortega et al., 2016; Deroche-Gamonet et al., 2018), and (2) CBD-treated mice could exhibit an enhancement of MAPK/CREB pathway signalling, congruent with an upregulated CB1R function in the hippocampus. To confirm these hypothesis it is crucial to find evidence of a MAPK/CREB pathway modulation that could link a putative CB1R upregulation with the hypothesized neural proliferation facilitation by CBD. Nevertheless, no studies have so far addressed the pro-neurogenic effects of CBD or its modulation of hippocampal MAPK/CREB pathway in drug-dependent animals.

In this context, the aim of the present study was to investigate whether CBD could modulate cocaine behavioural neuroadaptations using the intravenous self-administration paradigm, a compelling mouse model of cocaine intake, and to better understand the modulatory role of CBD on the signalling pathways underlying cocaine consumption. The possible effects elicited by CBD on anxiety-like responses and cognitive effects in drug-naïve mice were also investigated. In order to evaluate the role of CBD in modulating behavioural neuroadaptations to cocaine, we settled a subchronic CBD treatment to test its anxiolytic and cognitive effects. Then, we administered the CBD treatment in animals undergoing cocaine-related behavioural procedures (behavioural sensitization, conditioned place preference and self-administration), and we found that CBD attenuated cocaine-induced behavioural neuroadaptations, excluding behavioural sensitization. We have also proved that CBD modified CB1R expression and MAPK signalling in the hippocampus, and increased adult hippocampal neurogenesis in cocaine self-administering mice.

2. Materials and methods

2.1. Animals and drugs

Male CD1 mice (postnatal day 41–44) were purchased from Charles River (Barcelona, Spain). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were maintained in a 12-h light–dark cycle, in stable conditions of temperature (22 °C), with food and water *ad libitum*. The CD1 mouse strain was selected for its optimal sensitivity to the reinforcing and psychostimulating effects of cocaine (McKerchar et al., 2005). Four different sets of mice were used for the elevated plus maze/object recognition tests (n = 40), cocaine-induced sensitization (n = 24), conditioned place preference (n = 157) and self-administration (n = 72) experiments. All animal care and experimental protocols were approved by the PRBB-UPF Animal Ethics Committee, in line with European Community Council guidelines (2016/63/EU).

Cocaine HCl (0.75 or 10 mg/kg; Alcaliber S.A., Madrid, Spain) was daily prepared fresh and dissolved in 0.9% NaCl solution. CBD (5, 10, 20 and 30 mg/kg) was generously provided by Phytoplant Research S.L. (Córdoba, Spain). CBD was first mixed with 2% Tween-80 and then suspended by sonication (15 min) in 0.9% NaCl. CBD solution was prepared once and used along 10 days of treatment. For control groups, vehicle consisted in a solution of 2% Tween-80 in 0.9% NaCl. The volume of injections for all the drugs used in this study was 0.1 mL per 10 g of mouse body weight.

2.2. Drug administration protocols and experimental design

For the elevated plus maze, object recognition, cocaine-induced sensitization and conditioned place preference experiments, mice were treated with CBD (5, 10, 20 and 30 mg/kg) or vehicle by intraperitoneal (i.p.) injection for 10 consecutive days, and 5 days after the last CBD injection mice underwent behavioural testing. In the case of the self-administration experiments, mice received an i.p. administration of CBD 20 mg/kg just before each acquisition session, so in this set of experiments mice were treated in each session with CBD immediately before being placed in the operant chambers and also during 10 consecutive days. The CBD doses selected for this study were based on previous experimental studies (Ren et al., 2009; Gonzalez-Cuevas et al., 2018). The behavioural procedures were carried out 5 days after the last CBD exposure to evaluate the effects of a CBD sub-chronic treatment on neuroplasticity. For the self-administration experiment, behavioural timelines were more extended, and CBD was administered during the acquisition phase to ensure efficient treatment outcome. In this case, we cannot discard that the acute effects of CBD could also participate in the observed results. The progressive ratio test was

maintained 5 days after halting CBD treatment (as previous behavioural tests) to avoid the acute effects of CBD on this schedule of reinforcement and to maintain consistence between protocols.

Animals were randomly assigned to an experimental group. During the behavioural manipulations and data interpretation, researchers were blind to the treatment that each animal had received.

2.3. Elevated plus maze test

The elevated plus maze test was performed as reported (Gracia-Rubio et al., 2016), and it was carried out 5 days after the last CBD administration. Each mouse (n = 8) was placed in the centre of the maze for 5 min. The percentage of time spent into the open arms was measured using the Smart Software (Panlab s.l.u., Barcelona, Spain).

2.4. Novel object recognition task

The novel object recognition task used here was performed as described by Cantacorps et al. (2017), and started 24 h after the elevated plus maze test. First, CBD (5, 10, 20 and 30 mg/kg) (n = 8/dose) or mice pre-treated with vehicle (n = 8) were individually acclimatized to the box for 15 min. After 24 h, the animals could explore the maze for 10 min in the presence of either object A or object B (counterbalanced). The retention trial occurred 24 h after and the objects A and B were simultaneously placed in the open-field. The recognition index (%) was defined as $\frac{t_{novel\ object}}{(t_{novel\ object} + t_{familiar\ object})} \times 100$, being "*t*" the time each mice spent exploring an object (recorded using Smart Software, Panlab s.l.u., Barcelona, Spain).

2.5. Cocaine-induced conditioned place preference

Cocaine-induced conditioned place preference was assessed as previously described (López-Arnau et al., 2017). Experimental groups were the following: saline-vehicle, n = 27; saline-CBD 5 mg/kg, n = 11; saline-CBD 10 mg/kg, n = 22; saline-CBD 20 mg/kg, n = 12; saline-CBD 30 mg/kg, n = 12; cocaine-vehicle, n = 33; cocaine-CBD 5 mg/kg, n = 11; cocaine-CBD 10 mg/kg, n = 13; cocaine-CBD 20 mg/kg, n = 16; cocaine-CBD 30 mg/kg, n = 16. We used an unbiased conditioned place preference paradigm in which mice were placed in the central compartment and had free access to both compartments of the apparatus (Cibertec S.A., Madrid, Spain) for 18 min. During the conditioning phases (4 cocaine pairings, 8 days), mice received an i.p. injection of cocaine 10 mg/kg immediately before being placed into one of the two conditioning compartments for 20 min. On the alternate days, mice were treated with a saline injection and placed in the other compartment for 20 min. Control animals received saline every day. Data was represented as the difference of time spent between drug-paired and saline-paired compartments (*s*) on the testing day.

2.6. Cocaine-induced behavioural sensitization

Cocaine-induced behavioural sensitization was assessed as previously described (López-Arnau et al., 2017). Briefly, to sensitize locomotor responses induced by repeat cocaine treatment, mice (n = 12) were administered with cocaine (10 mg/kg) or saline and placed on actimetry boxes (LE8811R, Panlab, Barcelona, Spain) provided with 14 photobeam lasers (X and Y axes) for five consecutive sessions of 15 min. A cocaine challenge (10 mg/kg, 15 min) was administered 3 days after the last cocaine injection. Total locomotor activity was defined as the total number of photobeam crosses performed by an animal in a session.

2.7. Cocaine operant self-administration

Self-administration experiments were conducted as previously described in Soria et al. (2008) and López-Arnau et al. (2017). CBD-(20 mg/kg) and vehicle-treated mice (n = 36) were trained to self-administer cocaine (0.75 mg/kg/infusion) daily (2 h) during 10 consecutive days under fixed ratio 1. Surgical implantation of the catheter into the jugular vein was performed following anaesthetization with a mixture of ketamine hydrochloride (100 mg/kg; Imalgène1000, Lyon, France) and xylazine hydrochloride (20 mg/kg; Sigma Chemical Co., Madrid, Spain). The anaesthetics solution was injected in a volume of 0.15 mL/10 g body weight, i.p (Tourino et al., 2012). After surgery, mice were housed individually and allowed to recover for at least 3 days. During recovery, mice were treated daily with an analgesic (meloxicam 0.5 mg/kg, injected in a volume of 0.1 mL/10 g, i.p.) and an antibiotic solution (enrofloxacin 7.5 mg/kg, injected in a volume of 0.03mL/10 g, i.p.). The home cages were placed upon thermal blankets to avoid post-anaesthesia hypothermia.

2.7.1. Acquisition of operant cocaine taking

Active and inactive nose-pokes holes were assigned randomly. Cocaine was delivered in a 20 μl injection over 2 s via a syringe mounted on a microinfusion pump (PHM-100 A, Med-Associates, Georgia, VT, USA) connected to the mouse's intravenous catheter. Fixed ratio 1 session started with a cocaine priming infusion. When mice responded on the active hole, the stimulus lights lit up for 4s. Each infusion was followed by a 15s time-out period. Mice were considered to have acquired stable self-administration behaviour when the following criteria were met on 2 consecutive fixed ratio 1 sessions: a) 80% stability in reinforcements/infusions (the number of reinforces on each day deviated by $<$ 20% from the mean number of reinforces over the 2 consecutive days); b) $\geq$ 65% of responses were received at the active hole, and c) a minimum of 5 infusions on the active hole. After training, mice that met acquisition criteria (vehicle, n = 32; CBD, n = 23) were moved to a progressive ratio session, in which the response requirement to earn an injection escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The progressive ratio session was carried out because it allowed us to evaluate motivational functions (such as instrumental learning, execution of efforts and sustained engagement) closely related to the neuroadaptations underlying drug abuse (Randall et al., 2012; Richardson and Roberts, 1996). After completing the progressive ratio session, 9–10 animals of each treatment group were randomly selected for brain tissue extraction and biochemistry analysis.

2.7.2. Extinction and reinstatement of cocaine seeking

All animals that met the acquisition criteria underwent an extinction phase, after progressive ratio session, in which nosepokes in the active hole produced neither cocaine infusion nor stimulus light presentation (López-Arnau et al., 2017). Extinction sessions (2 h) were conducted once a day, 5 days/week until reaching the extinction criteria (performing less than 40% of the mean nosepokes given after having reached a stable fixed ratio 1 response). Twenty-four hours after, mice underwent a cocaine-primed reinstatement session, in which they were confined to operant chambers for 2 h immediately after receiving an i.p. cocaine injection (10 mg/kg). After the reinstatement session, five animals per group were randomly selected and were sacrificed and perfused for doublecortin immunohistochemistry experiments.

2.8. Western blotting and enzyme-linked immunosorbent assay

Seven days after CBD treatment in cocaine-naïve and self-administering mice (Fig. 2a), total protein extracts were isolated from whole hippocampus brain area (the exact number of samples is indicated in the correspondent figure legend). Hippocampal tissue harvest was performed 7 days after finishing CBD treatment to obtain data from the molecular and cellular markers at the same temporal point than the behavioural changes observed, once we can be sure that cocaine plasma levels were disappeared. This time lapse was also required for BrdU

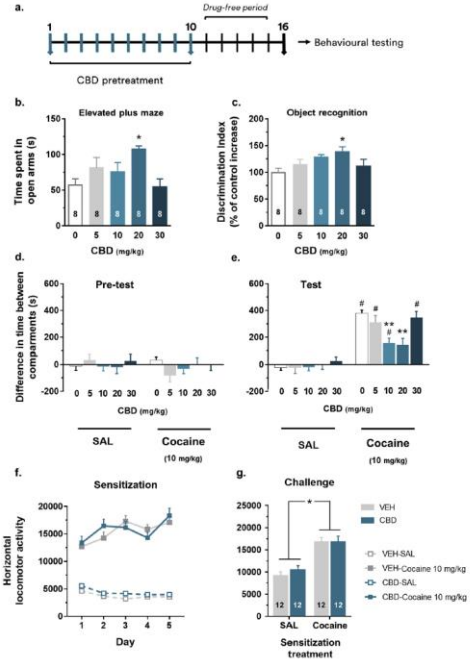

Fig. 1. Effects of CBD treatment in the elevated plus maze, object recognition and cocaine-induced conditioned place preference and behavioural sensitization. (a) Schematic representation of the CBD pre-treatment and the subsequent behavioural experiments. (b) Time spent in the open arms (s) of the elevated plus maze (n = 8/group) (Tukey, $^{*}p < 0.05$ vs control group). (c) Discrimination index in the object recognition test shown during the retention trial (15 min) (n = 8/group) (Tukey, $^{*}p < 0.05$ vs control group). (d, e) Difference in time spent in the cocaine-paired compartment compared to the saline-paired compartment in the same session, before and after the 10 mg/kg cocaine pairings (saline-vehicle, n = 27; saline-CBD 5 mg/kg, n = 11; saline-CBD 10 mg/kg, n = 22; saline-CBD 20 mg/kg, n = 12; saline-CBD 30 mg/kg, n = 12; cocaine 10 mg/kg-vehicle, n = 33; cocaine 10 mg/kg-CBD 5 mg/kg, n = 11; cocaine 10 mg/kg-CBD 10 mg/kg, n = 13; cocaine 10 mg/kg-CBD 20 mg/kg, n = 16; cocaine 10 mg/kg-CBD 30 mg/kg, n = 16) (Tukey, $^{**}p < 0.01$ *vs* cocaine 10 mg/kg-vehicle group; $\#p < 0.01$ *vs* respective saline-treated group). (f, g) Locomotor activity during each behavioural sensitization session and after the cocaine 10 mg/kg challenge (n = 12/group) (Tukey, $^{*}p < 0.05$ saline-*vs* cocaine 10 mg/kg-treated groups).

incorporation in still-young neurons that express the fate marker NeuN (Snyder et al., 2009).

The tissue was first homogenized in 600 μL of 4 °C lysis buffer [0.15 M NaCl, 1% TX-100, 10% glycerol, 1 mM EDTA, 50 mM TRIS pH = 7.4 and a phosphatase and protease inhibitor cocktail (Roche, Basel, Switzerland)]. Homogenates were centrifuged at 15,000 g for 15 min at 4 °C. Aliquots of resulting supernatants (total lysate) were stored at −80 °C until use for western blotting or ELISA determination.

2.8.1. Western blotting

Equal amounts of protein (20 μg) for each sample were mixed with loading buffer (153 mM TRIS pH = 6.8, 7.5% SDS, 40% glycerol, 5 mM EDTA, 12.5% 2-β-mercaptoethanol and 0.025% bromophenol blue) and loaded onto 10% polyacrylamide gels, and transferred to PVDF sheets (Immobilion-P, MERCK, Burlington, USA). Membranes were immunoblotted using the primary antibodies listed in Table 1, and then incubated for 1 h with their respective secondary fluorescent antibodies: goat anti-rabbit (1:2500, Rockland, PA, USA) or goat anti-mouse (1:2500, Abcam, Cambridge, UK). Protein expression was quantified using a Li-Cor Odyssey scanner (Li-Cor, Lincoln, USA).

2.8.2. ELISA determination

BDNF ELISA quantification was performed with the ELISA Emax Immunoassay system (Promega, Madrid, Spain), as in Moscoso-Castro et al. (2016), using Max-iSorp 96 well plates (Nunc, Denmark). The supernatant was acid-treated and then neutralized to increase the amount of detectable BDNF. BDNF levels were calculated as the percentage of the corresponding control normalized to the total amount of protein.

Data from both biochemical analyses was normalized to 100%, defined as the mean of the technical replicates in the control group.

2.9. Immunofluorescence

The neuronal proliferation-related markers were selected according to previous studies (Castilla-Ortega et al., 2017) and they were the following: (1) 5-bromo-2′-deoxyuridine (BrdU) to label the proliferating population that incorporated BrdU and started expressing the neuronal fate marker Neuronal Nuclei (NeuN), and (2) the microtubule-associated protein doublecortin used to label more mature neurons (up to four weeks), which may have started developing a differentiated neuronal morphology. Mice were treated three times with the BrdU stain

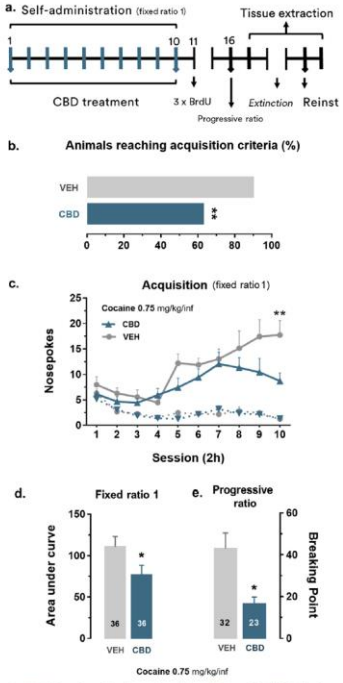

Fig. 2. CBD 20 mg/kg reduced cocaine-taking (0,75 mg/kg/inf) behaviour and breaking point values under a progressive ratio reinforcement schedule. **(a)** Schematic representation of the behavioural procedures carried out. **(b)** Difference in self-administration behaviour acquisition ratios between vehicle- and CBD-treated mice (Fisher's exact test, $^{**}p < 0.01$). **(c)** Nosepoke activations during the self-administration sessions under a fixed ratio 1 reinforcement schedule (n = 36). Dashed lines represent inactive nosepokes of the corresponding group (Tukey, $^{**}p < 0.01$ *vs* CBD-treated group active nosepokes). **(d)** Area under the curve of the active nosepokes performed during the whole fixed ratio 1 phase (Tukey, $^{*}p < 0.05$). **(e)** Breaking point achieved in a 2 h session under a progressive ratio reinforcement schedule (0,75 mg/kg/inf). Only animals that reached the acquisition criteria are represented (vehicle, n = 32; CBD, n = 23) (Tukey, $^{*}p < 0.05$).

(100 mg/kg, MERCK) within 24 h. Seven days later, mice were anaesthetized with pentobarbital (500 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), as in Gracia-Rubio et al. (2016). A mouse brain atlas (Paxinos and Franklin, 2004) was used to identify the anatomical location of the dentate gyrus (a minimum of 3 coronal sections per animal, evaluated bilaterally). Floating brain sections (30 μm-thick) were incubated in 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. Finally, BrdU-NeuN (n = 4) and doublecortin (n = 5) labelling was analysed by incubating sections overnight with the corresponding primary antibody (Table 1). Samples were then incubated for 2 h with a fluorescent secondary antibody, namely goat anti-rat IgG Alexa Fluor 488 (1:500; ThermoFisher, Barcelona, Spain) and goat anti-mouse IgG Alexa Fluor 555 (1:500; ThermoFisher). Sections were mounted on slides with Fluoroshield media (Sigma-Aldrich) and coverslipped for microscopy. Double-labelled images of the region of interest were obtained bilaterally using sequential laser scanning confocal microscopy (Leica SP2 and Zeiss LSM510). BrdU/NeuN + and doublecortin/Hoechst + neurons were quantified as the mean number of double-labelled body cells in each hemisphere per brain slice. Cells were counted using ImageJ software (NIH, Bethesda, MD, USA) by an observer who was blind to treatment.

2.10. Statistical analysis

Elevated plus maze and object recognition test's data was analysed using one-way analysis of variance (ANOVA) with the factor defined as *CBD treatment* ("VEH" and the corresponding "CBD dose" levels). We analysed the results of the conditioned place preference test, differences in protein phosphorylation and BrdU staining using two-way ANOVA analysis. For the conditioned place preference test, factors were designed as *CBD treatment* and *cocaine treatment*. In the protein phosphorylation studies, factors were defined as *CBD treatment* and *protein phosphorylation* or *subunit ratio*. BrdU staining factors were *CBD treatment* and *cocaine self-administration* (used levels: mice that underwent "self-administration" and "cocaine-naïve" mice). We calculated three-way ANOVA analysis to test the acquisition, extinction and reinstatement of the self-administration and the sensitization experiments. The factors for the self-administration acquisition and extinction data were defined as *CBD treatment, day of training* (repeated measure) and *nosepoke* (within-subjects factor, "active" and "inactive" levels). For the reinstatement, factors were defined as *CBD treatment, nosepoke* and *experimental phase* (repeated measure). Factors for the sensitization study were defined as *CBD treatment, day* (repeated measure) and *cocaine treatment*. To analyse the differences between treatments in the progressive ratio test, and CB1R and BDNF expressions Student t-test was used. When required, ANOVA analysis was followed by Tukey's post-hoc tests. We used the Fisher exact test to compare acquisition ratios of self-administration behaviour. The α-level of statistical significance was set at $p < 0.05$. Data were expressed as mean ± SEM. The exact group size for the individual experiments is shown in the corresponding figure legends. Statistical analyse were made using GraphPad Prism 7, La Jolla, USA.

3. Results

3.1. CBD pre-treatment reduces anxiety-like behaviour in the elevated plus maze and improves object recognition memory

One-way ANOVA calculation of the percentage of time spent in open arms showed a significant *CBD treatment* effect ($F_{4,31} = 3.94$; $p = 0{,}012$) (Fig. 1b). Tukey *post-hoc* test showed a higher percentage of time spent in the open arm in the CBD 20 mg/kg group than in the control group ($p = 0{,}022$). CBD 5, 10 and 30 mg/kg-treated groups showed no differences with the control group (Tukey, $p > 0{,}05$). The object recognition test was used to assess the effects of the CBD treatment on recognition memory, a hippocampal-dependent task. One-way ANOVA analysis yielded a significant effect of the *CBD treatment* factor ($F_{4,33} = 2.66$; $p = 0{,}049$) (Fig. 1c). *Post-hoc* analyses showed that CBD at 20 mg/kg improved object recognition performance compared to control group ($p = 0{,}0479$).

Table 1
List of the primary antibodies used for the immunohistochemistry and western blot studies.

Antibody	Description	Host	Dilution	Company	Item number
CB1	*cannabinoid receptor type 1*	Rabbit	1:1000	Frontiers Institute	#AB_2571591
CREB	*cAMP response element-binding protein*	Rabbit	1:500	MERCK	#04-767
Doublecortin	*also, lissencephalin-X*	Rabbit	1:2000	Abcam	#ab18723
$ERK_{1/2}$	*extracellular signal-regulated kinases* 1/2	Mouse	1:1000	Abcam	#ab54230
GluA1	*AMPA receptor subunit 1*	Rabbit	1:2000	MERCK	#ABN241
GluA2	*AMPA receptor subunit 2*	Rabbit	1:5000	MERCK	#AB1768-I
NeuN	*Neuronal Nuclei*	Mouse	1:1000	MERCK	#MAB377
pCREB	*Phosphorylated cAMP response element-binding protein*	Rabbit	1:1000	MERCK	#06-519
$pERK_{1/2}$	*Phosphorylated extracellular signal–regulated kinases* ½	Mouse	1:5000	Abcam	#ab50011
Tub	*Class III β-tubulin*	Mouse	1:5000	BD Pharmigen	#556321

3.2. Cocaine-induced conditioned place preference is impaired by CBD treatment

As shown in Fig. 1d, mice treated with vehicle or CBD (5, 10, 20 and 30 mg/kg) presented no initial unconditioned preference for any compartments of the conditioned place preference maze. Two-way ANOVA testing differences in the time spent in each compartment on the test day showed a significant effect for *CBD treatment* ($F_{4,176} = 5.77$; $p = 0{,}0002$), *cocaine treatment* ($F_{1,176} = 144.7$; $p = 0{,}0001$) and interaction between these factors ($F_{4,176} = 5.71$; $p = 0{,}0002$). Mice treated with CBD 10 (Tukey, $p = 0{,}0001$) and 20 mg/kg (Tukey, $p = 0{,}0001$) showed a reduced preference towards the cocaine-paired compartment (Fig. 1e).

Based on these results, we selected a CBD dose of 20 mg/kg for the next experiments.

3.3. Cocaine-induced locomotor sensitization is not affected by CBD treatment

Cocaine induced a hyperlocomotor response that increased equally in the CBD- and vehicle-treated groups (Fig. 1f). Three-way ANOVA analysis with repeated measures showed an effect of *day* ($F_{4,4} = 2.67$; $p = 0{,}032$), *cocaine treatment* ($F_{1,4} = 1035$; $p = 0{,}0001$) and interaction between these factors ($F_{4,4} = 8.2$; $p = 0{,}0001$); there was no significant effect for the *CBD treatment* ($F_{1,4} = 1.72$; $p = 0{,}191$). When mice were challenged three days after the last cocaine injection (Fig. 1g), there were no differences in cocaine-induced locomotor responses between CBD- and vehicle-treated mice (*cocaine treatment* factor $F_{1,44} = 40.17$; $p = 0{,}0001$ and *CBD treatment* factor $F_{1,44} = 0.34$; $p = 0{,}558$).

3.4. CBD treatment specifically disrupts the acquisition of cocaine self-administration behaviour

Vehicle- and CBD-treated mice were trained to self-administer 0,75 mg/kg/inf cocaine for 10 days under a fixed ratio 1 reinforcement schedule (Fig. 2a). CBD 20 mg/kg was administered immediately before the beginning of each self-administration session; the percentage of animals who met the acquisition criteria was lower in the CBD group (Fisher's exact test; $p = 0{,}0028$) (Fig. 2b).

The three-way ANOVA analysis (*CBD treatment* × *day of training* × *nosepoke*) showed significant effects for *CBD treatment* ($F_{1,9} = 14.02$, $p = 0{,}0002$) (Fig. 2c), *day of training* ($F_{9,71} = 6.47$; $p = 0{,}0001$), *nosepoke* ($F_{1,71} = 254.1$; $p = 0{,}0001$), and interaction between these factors (*training x nosepoke* $F_{9,71} = 8.34$; $p = 0{,}0001$). We found an interaction between *CBD treatment* and *nosepoke* ($F_{1,71} = 10.49$; $p = 0{,}0012$). *Post-hoc* comparisons revealed a difference between vehicle and CBD groups in cocaine intake at day 10 (Tukey; $p = 0{,}0041$). Moreover, the area under the curve of the whole fixed ratio 1 cocaine intake revealed that CBD-treated group consumed less cocaine during the whole procedure than VEH-treated group (Student's t-test; $t_{71} = 2.00$; $p = 0{,}0497$) (Fig. 2d). In the progressive ratio test (the number of active nosepokes given during the whole session), Student's t-test showed that CBD-treated mice displayed a significant decrease in breaking point values (Student's t-test; t53 = 2.89; p = 0,0055) (Fig. 2e).

Cocaine-seeking behaviour was extinguished in both groups as revealed by the three-way ANOVA analysis; *day of training* ($F_{9,15} = 3.2$; $p = 0{,}006$) and its interaction with *nosepoke* ($F_{9,15} = 2.15$; $p = 0{,}0086$)], without differences between treated groups (*CBD treatment;* $F_{1,15} = 0.09$; $p = 0{,}576$) (Fig. 3b). Two animals from each group did not extinguish cocaine-seeking behaviour. After extinction, animals were cocaine-primed to reinstate their cocaine-seeking behaviour (Fig. 3c). Nosepokes measures were analysed throughout the different experimental phases of which the self-administration procedure consisted. Only data from animals that finally reinstated was used for these analyses. The three-way ANOVA analysis showed significant effects for the *experimental phase* ($F_{1,15} = 8.47$; $p = 0{,}011$) and the *nosepoke* factors ($F_{1,15} = 27.7$; $p = 0{,}0001$). Although no differences in the *CBD pretreatment* factor were found ($F_{1,15} = 0{,}221$; $p = 0{,}645$), a significant *CBD pretreatment* x *experimental phase* interaction was observed ($F_{1,15} = 7.517$; $p = 0{,}015$). Compared to the active nosepokes given during the last extinction session (Fig. 3c), both groups reinstated its cocaine-seeking behaviour (Tukey, $p < 0{,}05$) and no differences were observed between vehicle- and CBD-treated groups in the reinstatement session (Tukey, $p > 0{,}05$).

3.5. CBD treatment increases MAPK-CREB activity and neuronal proliferation in the hippocampus, and reduces the GluA1/2 ratio in the STR after cocaine self-administration

As CBD 20 mg/kg specifically impaired the acquisition of the cocaine taking behaviour, we sought to determine whether CBD influenced the activation of hippocampus MAPK pathway and its downstream pathways that regulate the expression of the transcriptional (CREB) and neurotrophic (BDNF) factors, which are in turn responsible for the levels of neuronal proliferation in this brain area. Hence, following CBD treatment during self-administration acquisition, we investigated phosphorylation and expression levels of extracellular signal–regulated kinases 1/2 (ERK1/2), CREB, CB1R and BDNF (Fig. 4) parallel to neuronal proliferation (Fig. 5). The striatal AMPA receptor subunit 1/2 (GluA1/2) ratio was also measured at this time point. Additionally, we assessed neuronal differentiation (doublecortin) in the dentate gyrus 24 h after completing the cocaine-seeking behaviour reinstatement session.

After CBD treatment, CB1R expression was increased in the hippocampus (Student's *t*-test; $t_{10} = 3.24$; $p = 0{,}0088$) (Fig. 4b). Moreover, mice treated with CBD showed an increase in ERK1/2 phosphorylation (*CBD treatment,* $F_{1,30} = 7.98$; $p = 0{,}0083$; *protein phosphorylation*, $F_{2,30} = 3.4$, $p = 0{,}0463$; interaction, $F_{2,30} = 3.43$, $p = 0{,}0455$) (Fig. 4c). CREB phosphorylation was also upregulated by CBD (*CBD treatment,* $F_{1,30} = 4.74$, $p = 0{,}0374$; *protein phosphorylation*, $F_{2,30} = 3.94$, $p = 0.0302$ and interaction, $F_{2,30} = 4.67$, $p = 0{,}0171$)

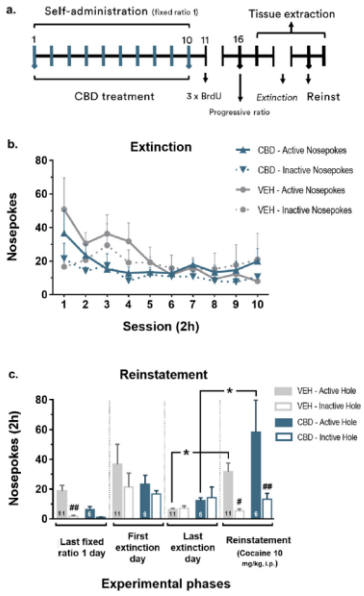

Fig. 3. CBD did not attenuate cocaine-seeking behaviour following extinction training phase and drug-induced reinstatement. **(a)** Schematic representation of the behavioural procedures carried out. **(b)** Nosepoke activations during the extinction training sessions (vehicle, n = 32; CBD, n = 23). We represent the mean number of days that cocaine-seeking behaviour took to extinguish. **(c)** Cocaine-induced (10 mg/kg, i.p.) reinstatement of self-administration behaviour in a 2 h session, 24 h after reaching the extinction criteria (Tukey, $*p < 0.05$) (Tukey, $\#p < 0.05$, $\#\#p < 0.01$ *vs* active nosepokes of the same group in the same experimental phase) (vehicle, n = 9; CBD, n = 4). Only animals that reached the extinction criteria are represented.

(Fig. 4d). Moreover, ELISA analyses revealed a significant increase in BDNF expression in the hippocampus in the CBD-treated group (Student's *t*-test; $t_{13} = 2.81$; $p = 0{,}0147$) (Fig. 4e). In CBD-treated mice, the STR GluA1/2 ratio was found to be reduced [*subunit ratio* ($F_{2,30} = 4.28$; $p = 0{,}0231$); *treatment* ($F_{1,30} = 2.3$; $p = 0{,}136$), with an interaction between these factors, ($F_{2,30} = 4.19$; $p = 0{,}0247$)]. This significant interaction allowed us to confirm by *post-hoc* comparisons that the GluA1/2 ratio was reduced by CBD (Tukey, $p = 0{,}0496$), unlike the net levels of GluA1 (Tukey, $p = 0{,}359$) or GluA2 (Tukey, $p = 0{,}421$) subunits (Fig. 4f).

To compare dentate gyrus levels of neuronal proliferation in vehicle- and CBD-treated mice, we administered BrdU (100 mg/kg) i.p. during the drug-free period, and then counted the number of BrdU/NeuN stained cells. To ascertain the effects of cocaine self-administration and CBD on neuronal proliferation, we also compared these cell counts to those in a group of mice that received the CBD treatment in their home cages, without submitting them to cocaine self-administration. We performed a two-way ANOVA analysis with *CBD treatment* and *cocaine self-administration* as main factors, and found that were significant (*CBD treatment*, $F_{1,14} = 47.4$, $p = 0{,}0001$ and *cocaine self-administration*, $F_{1,14} = 10.08$, $p = 0{,}0067$) (Fig. 5a), but that there was no interaction between them ($F_{1,14} = 0.17$, $p = 0{,}684$). Finally, Student's *t*-test showed that doublecortin expression was higher in CBD-treated mice than vehicle-treated animals (Student's *t*-test; $t_8 = 3.55$; $p = 0{,}0075$) (Fig. 5b).

4. Discussion

Our results show that repeated treatment with CBD may reduce the cocaine rewarding and reinforcing effects as modelled in the conditioned place preference and self-administration paradigms respectively. However, behavioural sensitization and drug-induced reinstatement of cocaine-seeking behaviour remained unaltered. In parallel, the GluA1/2 ratio was lower in the STR of CBD-treated mice. Our results also document a reduction of adult hippocampal neurogenesis after cocaine voluntary intake in CBD-treated mice. Related to the pro-neurogenic effect of CBD (Campos et al., 2013), our results show an upregulated CB1R expression in the hippocampus. Congruent to our findings, CBD treatment increased MAPK-CREB signalling and BDNF expression in the hippocampus. Together, these results deepen in the available knowledge concerning CBD actions in cocaine consuming animals along two new evidences: (**1**) that CBD treatment enhanced hippocampal neural proliferation concomitantly to a MAPK/CREB pathway upregulation after voluntary cocaine consumption attenuation despite (**2**) that not all the dimensions of the behavioural repertoire reflecting the abusive likelihood of cocaine were affected.

4.1. CBD treatment anxiolytic and cognitive properties

We show that the repeated CBD treatment used here produced anxiolytic effects in the elevated plus maze test. CBD is well known to produce an efficient anxiolytic response, especially when tested in this model (Lee et al., 2017). An intriguing issue of the anxiolytic profile reported here, and in the previous literature, is the observed effect in bell-shaped dose-response curve. In previous studies, the CBD doses varies among treatment schedules, administration routes, and animal species including mice (Marinho et al., 2015; Onaivi et al., 1990), rats (Guimarães et al., 1990), zebra fish (Nazario et al., 2015) and humans (Zuardi et al., 2017). The most plausible interpretation for that fact is the implication of multiple pharmacological mechanisms in the same observed effect, especially considering the pharmacological profile of CBD (Campos et al., 2012). Among those possible mechanisms, previous studies have pointed out the possibility of 5-$HT_1$$_A$R agonism (Marinho et al., 2015), CB1R modulation (Casarotto et al., 2010) and increased cell proliferation and neurogenesis (Campos et al., 2013; Schiavon et al., 2016). In agreement with these previous studies, our results show that the CBD dose able to produce anxiolytic effects induced also neural proliferation and CB1R increments in cocaine-naïve mice.

Results from the object recognition test indicate that CBD increased the discrimination index of the novel object, a behavioural correlate of declarative memory (Cohen and Stackman, 2015). It is noteworthy that previous studies using CBD have consistently observed similar cognitive effects in the object recognition test but only under vulnerability conditions such as amyloid-β aggregation (Cheng et al., 2014), previous chronic Δ^9-tetrahydrocannabinol treatment (Murphy et al., 2017), prenatal infection (Osborne et al., 2017) or new-born hypoxia-ischemia (Pazos et al., 2012). To our knowledge, this is a first-time report of an enhancing effect of CBD on object recognition discrimination index in control, healthy animals. The reason by which other studies did not find such cognitive effect under standard conditions may be elusive, but differences between treatments schedules could explain these

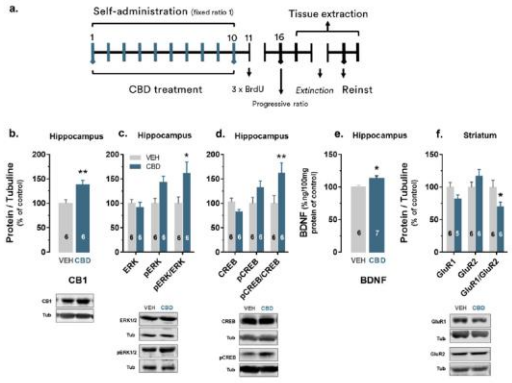

Fig. 4. CBD 20 mg/kg reduced striatal GluA1/2 ratio and upregulated MAPK-CREB pathway activity in the hippocampus when administered before each self-administration session. **(a)** Schematic representation of the behavioural procedures carried out. **(b, c, d)** Western blot analyses of CB1R, ERK/pERK and CREB/pCREB in the hippocampus of mice treated with vehicle or CBD (Tukey, $*p < 0.05$, $**p < 0.01$ vs vehicle) (n = 6). **(e)** BDNF protein levels in the hippocampus of CBD- and vehicle-treated animals, measured by ELISA (Student's *t*, $*p < 0.05$ vs vehicle) (vehicle, n = 6; CBD, n = 7). **(f)** Western blot analysis of striatal GluA1/2 ratio in mice treated with vehicle or CBD (Tukey, $*p < 0.05$ vs vehicle) (n = 6). The numbers in the bars represent the number of individuals in that group. The lower panels show representative fluorescence immunoblots.

divergences. For instance, Cheng et al. (2014) treated mice with CBD 20 mg/kg, but they prolonged treatment during 8 weeks, while conducting behavioural testing, thus animals received larger amount of CBD than in this study (20 mg/kg, 10 days). The capability of the cannabinoid Delta-9-tetrahydrocannabinol to be stored in fat reservoirs and then released back into blood plasma, producing long-lasting plasma concentration levels (Gunasekaran et al., 2009), raises the question of whether other phytocannabinoid compounds could build steady state plasma concentrations as well. Consroe et al. (1991) demonstrated that CBD had an elimination half-life of 2–5 days after a chronic treatment (10 mg/kg, 6 weeks) in humans and Gonzalez-Cuevas et al. (2018) reported that CBD (15 mg/kg) disappeared from brain tissue before the third day after the CBD administration. Based on this previous work, we consider that in our experiments CBD was metabolized and eliminated from the organism before the behavioural tests, although no pharmacokinetic tests were developed in this study.

Intriguingly, the anxiolytic and cognitive effects induced by CBD were observed at the same drug dose, showing a similar dose-response pharmacological profile. This issue raises the possibility for a linked effect between both behavioural outcomes. Evidence shows that cognitive tests performance can be related to anxiety-like responses (for an extensive review see Moran, 2016 and Owens et al., 2014). A negative correlation has been consistently reported between levels of anxiety and cognitive performance. In our study, it could be argued that vehicle-treated mice performed the novel object recognition in an elevated anxiety-like state (performed under stressful condition in the open field), thus showing a non-optimal cognitive performance. Given that the CBD treatment showed efficient anxiolytic properties, the improved cognitive performance of the CBD-treated group could be due to a lower anxiety state during testing. However, a specific mechanistic effect of CBD on both cognitive and anxiety-responses was not addressed in the present study and further studies are needed to clarify the possible link between these effects.

4.2. CBD treatment effects on cocaine-induced conditioned place preference and behavioural sensitization

CBD 10 and 20 mg/kg reduced cocaine-induced rewarding effects in the conditioned place preference paradigm. This effect is consistent with previous findings showing that CBD (10 mg/kg) disrupts reconsolidation of cocaine-associated conditioned place preference memories (de Carvalho and Takahashi, 2017), and facilitates extinction of cocaine-induced conditioned place preference (Parker et al., 2004). There are no previous findings of a CBD treatment targeting the acquisition of the learning association necessary for the induction of cocaine conditioned place preference, meaning that comparisons across studies are still limited to the learning mechanisms potentially targeted with CBD. Nonetheless, our results suggest that CBD can modulate different learning mechanisms required for cocaine-induced conditioned place preference acquisition, extinction and reconsolidation.

We observed that CBD attenuates certain behavioural adaptations after repeated cocaine, while others remained unaltered. Thus, we found that CBD does not affect cocaine behavioural sensitization in contrast with previous studies showing attenuated amphetamine-induced sensitization in rats (Renard et al., 2016). Note that, while we administered CBD via the i.p. route, Renard et al. (2016) microinfused CBD directly into the nucleus accumbens. CBD is known to have poor bioavailability (Devinsky et al., 2014), which could explain why systemic CBD administration results in weaker effects than intra-accumbens injections. However, given the lack of studies addressing the protective effects of CBD over cocaine-induced sensitization, it is still not possible to rule out the possibility that a drug-specific mechanism is producing the differences between Renard's et al. (2016) and this study. The lack of effect of an experimental manipulation on the sensitization process is often interpreted as an indicator that such manipulation does not modify the ability of the drug to interact with the mesolimbic dopaminergic system (Berridge and Robinson, 2016). Our behavioural sensitization results after CBD treatment seem to agree with such statement. Accordingly, Wu and French (2000) demonstrated that systemically administered CBD had neither excitatory nor inhibitory effects on spontaneously recorded VTA dopaminergic neuronal activity levels. Nevertheless, the lack of effect over the cocaine-induced actions in the mesolimbic dopaminergic system would not impede CBD to influence cocaine's reinforcing potential by other pharmacological mechanisms. Although no specific experiments have been carried out to elucidate the mechanisms by which CBD 10 and 20 mg/kg attenuated cocaine-induced conditioned place preference, we present evidence that the CBD treatment here used produced an increase of adult hippocampal neurogenesis in both cocaine-exposed and -naïve mice. Increases in adult hippocampal neurogenesis have been proposed as a

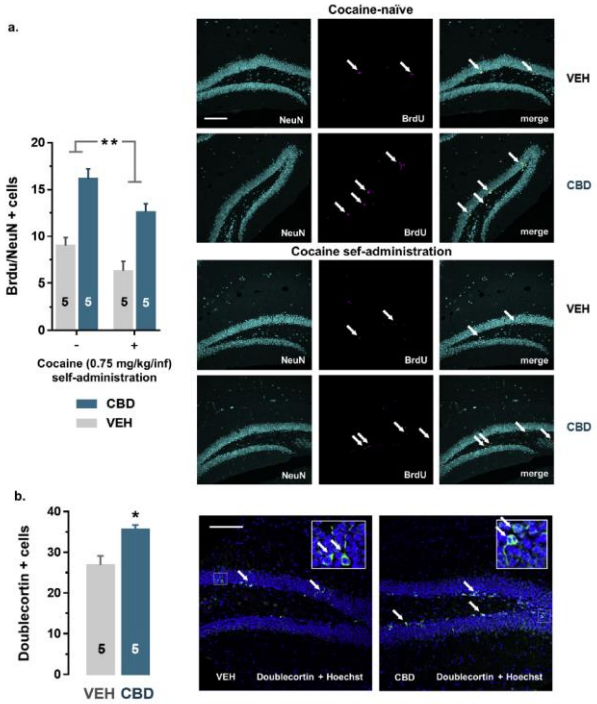

Fig. 5. CBD 20 mg/kg increased BrdU/NeuN double-labelling in the dentate gyrus of cocaine-naïve and cocaine-consuming (0,75 mg/kg/inf) mice. (a) Confocal sections of the dentate gyrus, showing immunofluorescence for the neuronal marker NeuN (cyan), the cell proliferation marker BrdU (purple), and its colocalization (yellow). Three injections of BrdU 100 mg/kg were i.p. administered 24 h after the last fixed ratio 1 self-administration session (0,75 mg/kg/inf). The left panel summarizes data represented as means ± SEM of BrdU/NeuN double-labelled cells (n = 5). Scale bar = 150 μm. White arrows indicate representative cases of BrdU/NeuN colocalization. (b) Confocal sections of the dentate gyrus, showing immunofluorescence for doublecortin (green) and Hoechst (blue) in animals treated with CBD or vehicle that underwent extinction and reinstatement of cocaine-seeking behaviour. Left panel summarizes data represented as means ± SEM of doublecortin-labelled cells (n = 4) (Student's *t*, $^{*}p < 0.05$ *vs* vehicle). Scale bar = 150 μm. White arrows indicate representative cases of doublecortin positive neurons. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mechanism to mitigate cocaine-induced conditioned place preference (Castilla-Ortega et al., 2016) by enhancing hippocampal plasticity (Kirby et al., 2015) and leading to beneficial changes in cortical-striatal network function (Chambers, 2013).

4.3. CBD treatment modulation of cocaine intake, progressive ratio breaking point and drug-induced reinstatement of cocaine-seeking behaviour

Previous studies have evaluated the efficacy of CBD to reduce cocaine seeking, with incongruent results (Gonzalez-Cuevas et al., 2018; Mahmud et al., 2017). We show that CBD 20 mg/kg is able to reduce cocaine taking under a fixed ratio 1 reinforcement schedule. Unlike Gonzalez-Cuevas et al. (2018) and Mahmud et al. (2017), we administered CBD during the acquisition phase of the self-administration to evaluate the impact of this cannabinoid in the cocaine taking behaviour and found that it was indeed reduced. Measures of responding under a fixed ratio 1 reinforcement schedule are considered a measure of the motivational consummatory phase with unique neuropsychological implications (Kelley et al., 2005), different from those of the approximatory or "appetitive" phase (for an extensive review, see Roberts et al., 2013). Within this theoretical framework, we remark the potential of CBD treatment to attenuate cocaine consummatory responses

in subjects undergoing acquisition and stabilization of a compulsive pattern of cocaine use.

While cocaine compulsive seeking was robustly reduced in a progressive ratio test, cocaine seeking under drug-relapse conditions remained unaffected. Two possible explanations could justify these apparent discrepancies. First, CBD-induced changes remained effective only until the progressive ratio test. After that, CBD-associated neuroplasticity disappeared or become irrelevant to cocaine-seeking behaviour, as observed in the reinstatement session's measurements. A second explanation arises considering the way to induce reinstatement. In this case, reinstatement was accomplished after giving a cocaine 10 mg/kg priming injection. Instead, Gonzalez-Cuevas et al. (2018) demonstrated an attenuation of cocaine seeking using cue- and stress-induced reinstatement procedures. In their study, drug-induced reinstatement was not evaluated. Note that the neurobiological circuitry underlying drug-seeking reinstatement vary depending on the source used to produce relapse (Bossert et al., 2013), what can explain the difference between the negative findings of our study and the attenuation of cue- and stress-induced reinstatement in Gonzalez-Cuevas et al. (2018). In summary, self-administration results do not seem to point CBD as a new treatment for cocaine abuse. The CBD regime of treatment used in this study did not attenuate cocaine reinstatement and consequently, CBD seems not to represent a putative pharmacological tool to tackle cocaine relapse in cocaine-abstinent subjects. Our study support, however, that the CBD treatment here investigated could be useful as an adjuvant medication in an agonist replacement therapy for cocaine dependence. Replacement therapies for cocaine abuse have been proposed during the last decade as an alternative therapeutic strategy to manage cocaine addiction (Negus and Henningfield, 2015). Although such strategies are neither validated nor approved, evidence points for using alternative dopaminergic agents to reduce cocaine craving underlying drug relapse (Czoty et al., 2012; Mariani et al., 2012). In this sense, CBD could reduce the compulsive and escalating intake patterns of replacement psychostimulants to cocaine. Therefore, the protective effects of CBD here reported open the possibility of using this phytocannabinoid to deal with the reinforcing potential of the replacement compound.

Based on the self-administration results, we suggest that the CBD actions do not seem directly modulate dopaminergic neurotransmission in the mesocorticolimbic system. Based on Roberts et al. (2013) revision of the issue, dopamine agents tend to oppositely modify cocaine intake and cocaine seeking measures. That is, experimental manipulations that directly interact with the dopamine system usually increase drug intake while reduce approximation response (De Wit and Wise, 1977; Depoortere et al., 1993; Hubner and Moreton, 1991; Yokel and Wise, 1975), or vice versa (Loh et al., 1992; Roberts and Vickers, 1984). However, other non-dopaminergic pharmacological manipulations produce no clear relationship between cocaine-taking and -seeking outcomes (España et al., 2010; Lin et al., 2012; McGregor et al., 1993). Given that CBD partially changed cocaine-taking and -seeking (as measured in the progressive ratio test) in the same direction, our results point out that the CBD effects observed here seem to be regulated by a non-dopaminergic mechanism.

Even so, pharmacological treatments can alter cocaine-induced mesolimbic maladaptations without directly interfering with the dopamine system (Loweth et al., 2014). AMPA receptor subunits redistribution is fundamental to synaptic plasticity throughout extensive brain areas for learning and memory functions. Furthermore, changes in GluA1/2 ratio can be associated with addiction-like behaviours (Wolf, 2016). In this line, previous studies have documented the ability of CBD to modify AMPA receptor subunits conformation in heroin-seeking rats (Ren et al., 2009), demonstrating its pharmacological potential to reduce some drug-associated neuroadaptations. In agreement, we also describe a reduction in striatal GluA1/2 AMPA receptor subunits ratio in CBD-treated, cocaine-consuming mice.

4.4. CBD changes on CB1R and BDNF expression, MAPK/CREB pathway phosphorylation and neural progenitor proliferation in the hippocampus of cocaine self-administering animals

We wanted to identify the neuroplastic changes present in CBD-treated, cocaine-consuming animals. Suppression of hippocampal neurogenic proliferation appears to be a common consequence of cocaine exposure (Deroche-Gamonet et al., 2018). Therefore, as no studies have assessed adult hippocampal neurogenesis in CBD-treated, drug-exposed mice, we wanted to test if the commonly observed reduction in hippocampal neural proliferation would be attenuated by CBD. Indeed, we observed an important increase of BrdU/NeuN staining in the dentate gyrus of CBD-treated, cocaine-consuming mice. However, this increase was not necessarily related to cocaine consumption, as cocaine-naïve mice did also express higher levels of BrdU/NeuN staining after CBD treatment. The lack of interaction between CBD treatment and cocaine self-administration in hippocampal neural proliferation probably means that CBD would not modulate the same mechanisms by which cocaine intake depletes adult hippocampal neurogenesis. In this regard, various drug-related anti-neurogenic mechanisms have been proposed, as oxidative stress or mitochondrial dysfunction (Cunha-Oliveira et al., 2008). Instead, CBD shall increase adult hippocampal neurogenesis by other means, probably by increasing CB1R neurotransmission in the hippocampus as proposed (Campos et al., 2013). Moreover, we consider that such mechanisms differed in its efficiency to modulate hippocampal neuronal proliferation, since CBD treatment produced a stronger effect on BrdU/NeuN cell count changes than cocaine consumption, as revealed in the statistical analysis. Accordingly to this hypothesis, we found that CBD significantly increased CB1R expression in the hippocampus, a receptor that positively regulates adult neurogenesis in this area (Prenderville et al., 2015). While it is not possible to determine how CBD caused this increase, previous studies have reported similar changes in CB1R expression after CBD administration (Ren et al., 2009; Viudez-Martínez et al., 2017). Even further, our data showed higher ERK1/2 (MAPK) and CREB phosphorylation, and enhanced BDNF expression in CBD-treated mice. Congruently, activation of CB1R is known to induce phosphorylation of the MAPK-CREB pathway (Mallipeddi et al., 2017), which in turn facilitates gene expression contributing to cell proliferation (Ortega-Martínez, 2015) and BDNF activity (Zhang et al., 2016). In line of this evidence, we propose that CBD may increase CB1R expression in the hippocampus resulting in an upregulation of the downstream MAPK/CREB pathway. Finally, MAPK/CREB pathway upregulation would translate into greater gene expression facilitation and BDNF concentration contributing to the increased neural cell proliferation here observed by immunohistochemistry.

Unfortunately, the complex pharmacology of CBD remains poorly understood, so any possible interpretation of data is likely to be incomplete. For instance, we observe an increase in the number of doublecortin-positive cells in the dentate gyrus of CBD-treated mice 24 h after the reinstatement session, and this marker of neuronal differentiation has been linked to attenuated cocaine-primed reinstatement (Deschaux et al., 2014). However, in this study, increased neuronal differentiation was not accompanied by lower cocaine-primed reinstatement. Besides, we cannot rule out the possibility that non-endocannabinoid neurotransmitter systems participate in the observed effects. The previous work of Zhang et al. (2016) showed that 5-HT1$_A$R activation also leads to CREB-mediated neurogenesis, such that CBD could also exert its pro-neurogenic actions through 5-HT1$_A$R activation (Russo et al., 2005).

5. Conclusion

We prove that CBD reduces cocaine voluntary consumption and progressive ratio breaking point in the self-administration paradigm, but it has no effect on cocaine-induced reinstatement. We extend the

compelling evidence supporting the pro-neurogenic properties of CBD to cocaine consuming animals. Moreover, we describe a series of molecular changes after cocaine self-administration related to the pro-neurogenic effects of CBD treatment. As previously described, CBD increase CB1R levels in the hippocampus. Accordingly, we document an upregulation induced by CBD of hippocampal MAPK/CREB pathway paralleled with an increase of the neurotrophic factor BDNF expression. Finally, we report a reduction in the striatal GluA1/2 AMPA receptor subunit ratio in agreement to the attenuation of cocaine voluntary consumption. The limitations of this study highlight the need to deepen our understanding of CBD-induced neurobiological effects to fully understand the therapeutic potential actions of this phytocannabinoid.

Acknowledgements

This work was supported by Ministerio de Economía y Competitividad (grant number SAF2016-75966-R-FEDER), by the European Union's Horizon 2020 research and innovation programme 2014–2020 under grant agreement no 634143. M.A.L. received FPU grant from the Ministerio de Economía y Competitividad (15/02492). A.C-Z was granted (276577) thanks to CONACYT fellowship programme (México). The Department of Experimental and Health Sciences (UPF) is an "Unidad de Excelencia María de Maeztu" funded by the MINECO (Ref. MDM-2014-0370). Authors thank to Phytoplant Research S.L. for providing cannabidiol compound. The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2018.09.043.

References

Berridge, K.C., Robinson, T.E., 2016. Liking, wanting, and the incentive-sensitization theory of addiction. Am. Psychol. 71, 670–679. https://doi.org/10.1037/amp0000059.

Bossert, J.M., Marchant, N.J., Calu, D.J., Shaham, Y., 2013. The reinstatement model of drug relapse: recent neurobiological findings, emerging research topics, and translational research. Psychopharmacology (Berlin) 229, 453–476. https://doi.org/10.1007/s00213-013-3120-y.

Campos, A.C., Fogaça, M.V., Scarante, F.F., Joca, S.R.L., Sales, A.J., Gomes, F.V., Sonego, A.B., Rodrigues, N.S., Galve-Roperh, I., Guimarães, F.S., 2017. Plastic and neuroprotective mechanisms involved in the therapeutic effects of cannabidiol in psychiatric disorders. Front. Pharmacol. 8, 269. https://doi.org/10.3389/fphar.2017.00269.

Campos, A.C., Moreira, F.A., Gomes, F.V., Del Bel, E.A., Guimarães, F.S., 2012. Multiple mechanisms involved in the large-spectrum therapeutic potential of cannabidiol in psychiatric disorders. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 3364–3378. https://doi.org/10.1098/rstb.2011.0389.

Campos, A.C., Ortega, Z., Palazuelos, J., Fogaça, M.V., Aguiar, D.C., Díaz-Alonso, J., Ortega-Gutiérrez, S., Vázquez-Villa, H., Moreira, F.A., Guzmán, M., Galve-Roperh, I., Guimarães, F.S., 2013. The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system. Int. J. Neuropsychopharmacol. 16, 1407–1419. https://doi.org/10.1017/S1461145712001502.

Cantacorps, L., Alfonso-Loeches, S., Moscoso-Castro, M., Cuitavi, J., Gracia-Rubio, I., López-Arnau, R., Escubedo, E., Guerri, C., Valverde, O., 2017. Maternal alcohol binge drinking induces persistent neuroinflammation associated with myelin damage and behavioural dysfunctions in offspring mice. Neuropharmacology 123, 368–384. https://doi.org/10.1016/j.neuropharm.2017.05.034.

Casarotto, P.C., Gomes, F.V., Resstel, L.B.M., Guimarães, F.S., 2010. Cannabidiol inhibitory effect on marble-burying behaviour: involvement of CB1 receptors. Behav. Pharmacol. 21, 353–358. https://doi.org/10.1097/FBP.0b013e32833b33c5.

Castilla-Ortega, E., Ladrón de Guevara-Miranda, D., Serrano, A., Pavón, F.J., Suárez, J., Rodríguez de Fonseca, F., Santín, L.J., 2017. The impact of cocaine on adult hippocampal neurogenesis: potential neurobiological mechanisms and contributions to maladaptive cognition in cocaine addiction disorder. Biochem. Pharmacol. 141, 100–117. https://doi.org/10.1016/j.bcp.2017.05.003.

Castilla-Ortega, E., Serrano, A., Blanco, E., Araos, P., Suarez, J., Pavon, F.J., Rodriguez de Fonseca, F., Santin, L.J., 2016. A place for the hippocampus in the cocaine addiction circuit: potential roles for adult hippocampal neurogenesis. Neurosci. Biobehav. Rev. 66, 15–32. https://doi.org/10.1016/j.neubiorev.2016.03.030.

Center for Behavioral Health Statistics and Quality, 2015. Behavioral Health Trends in the United States: Results from the 2014 National Survey on Drug Use and Health. (HHS Pulication No. SMA 15-4927, NSDUH Ser. H-50. 64.

Chambers, R.A., 2013. Adult hippocampal neurogenesis in the pathogenesis of addiction and dual diagnosis disorders. Drug Alcohol Depend. 130, 1–12. https://doi.org/10.1016/j.drugalcdep.2012.12.005.

Chauvet, C., Nicolas, C., Thiriet, N., Lardeux, M.V., Duranti, A., Solinas, M., 2015. Chronic stimulation of the tone of endogenous anandamide reduces cue- and stress-induced relapse in rats. Int. J. Neuropsychopharmacol. 18 pyu025-pyu025. https://doi.org/10.1093/ijnp/pyu025.

Cheng, D., Low, J.K., Logge, W., Garner, B., Karl, T., 2014. Chronic cannabidiol treatment improves social and object recognition in double transgenic APPswe/PS1ΔE9 mice. Psychopharmacology (Berlin) 231, 3009–3017. https://doi.org/10.1007/s00213-014-3478-5.

Cohen, S.J., Stackman Jr., R.W., 2015. Assessing rodent hippocampal involvement in the novel object recognition task. A review. Behav. Brain Res. 285, 105–117. https://doi.org/10.1016/j.bbr.2014.08.002.

Consroe, P., Kennedy, K., Schram, K., 1991. Assay of plasma cannabidiol by capillary gas chromatography/ion trap mass spectroscopy following high-dose repeated daily oral administration in humans. Pharmacol. Biochem. Behav. 40, 517–522.

Cunha-Oliveira, T., Rego, A.C., Oliveira, C.R., 2008. Cellular and molecular mechanisms involved in the neurotoxicity of opioid and psychostimulant drugs. Brain Res. Rev. 58, 192–208. https://doi.org/10.1016/j.brainresrev.2008.03.002.

Czoty, P.W., Stoops, W.W., Rush, C.R., 2016. Evaluation of the development of medications for cocaine use disorder: a review of translational preclinical, human laboratory, and clinical trial research. Pharmacol. Rev. 68, 533–562. https://doi.org/10.1124/pr.115.011668.

Czoty, P.W., Gould, R.W., Martelle, J.L., Nader, M.A., 2012. Prolonged attenuation of the reinforcing strength of cocaine by chronic d-amphetamine in rhesus monkeys. Neuropsychopharmacology 36, 539–547. https://doi.org/10.1038/npp.2010.185.

de Carvalho, C.R., Takahashi, R.N., 2017. Cannabidiol disrupts the reconsolidation of contextual drug-associated memories in Wistar rats. Addict. Biol. 22, 742–751. https://doi.org/10.1111/adb.12366.

De Wit, H., Wise, R.A., 1977. Blockade of cocaine reinforcement in rats with the dopamine receptor blocker pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. Can. J. Psychol. 31, 195–203.

Depoortere, R.Y., Li, D.H., Lane, J.D., Emmett-Oglesby, M.W., 1993. Parameters of self-administration of cocaine in rats under a progressive-ratio schedule. Pharmacol. Biochem. Behav. 45, 539–548.

Deroche-Gamonet, V., Revest, J.-M., Fiancette, J.-F., Balado, E., Koehl, M., Grosjean, N., Abrous, D.N., Piazza, P.V., 2018. Depleting adult dentate gyrus neurogenesis increases cocaine-seeking behavior. Mol. Psychiatr. https://doi.org/10.1038/s41380-018-0038-0.

Deschaux, O., Vendruscolo, L.F., Schlosburg, J.E., Diaz-Aguilar, L., Yuan, C.J., Sobieraj, J.C., George, O., Koob, G.F., Mandyam, C.D., 2014. Hippocampal neurogenesis protects against cocaine-primed relapse. Addict. Biol. 19, 562–574. https://doi.org/10.1111/adb.12019.

Devinsky, O., Cilio, M.R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., Katz, R., Di Marzo, V., Jutras-Aswad, D., Notcutt, W.G., Martinez-Orgado, J., Robson, P.J., Rohrback, B.G., Thiele, E., Whalley, B., Friedman, D., 2014. Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. Epilepsia 55, 791–802. https://doi.org/10.1111/epi.12631.

España, R.A., Oleson, E.B., Locke, J.L., Brookshire, B.R., Roberts, D.C.S., Jones, S.R., 2010. The hypocretin-orexin system regulates cocaine self-administration via actions on the mesolimbic dopamine system. Eur. J. Neurosci. 31, 336–348. https://doi.org/10.1111/j.1460-9568.2009.07065.x.

European Monitoring Centre for Drugs and Drug Addiction, 2016. European Drug Report 2016: Trends and Developments, European Monitoring of Drugs and Drugs Addiction. . https://doi.org/10.2810/88175.

Everitt, B.J., Giuliano, C., Belin, D., 2018. Addictive behaviour in experimental animals: prospects for translation. Philos. Trans. R. Soc. B: Biol. Sci. 373, 1742. https://doi.org/10.1098/rstb.2017.0027.

Gerdeman, G.L., Schechter, J.B., French, E.D., 2008. Context-specific reversal of cocaine sensitization by the CB1 cannabinoid receptor antagonist rimonabant. Neuropsychopharmacology 33, 2747–2759. https://doi.org/10.1038/sj.npp.1301648.

Gonzalez-Cuevas, G., Martin-Fardon, R., Kerr, T.M., Stouffer, D.G., Parsons, L.H., Hammell, D.C., Banks, S.L., Stinchcomb, A.L., Weiss, F., 2018. Unique treatment potential of cannabidiol for the prevention of relapse to drug use: preclinical proof of principle. Neuropsychopharmacology 1–10. https://doi.org/10.1038/s41386-018-0050-8.

Gracia-Rubio, I., Moscoso-Castro, M., Pozo, O.J., Marcos, J., Nadal, R., Valverde, O., 2016. Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 65, 104–117. https://doi.org/10.1016/j.pnpbp.2015.09.003.

Guimarães, F.S., Chiaretti, T.M., Graeff, F.G., Zuardi, A.W., 1990. Antianxiety effect of cannabidiol in the elevated plus-maze. Psychopharmacology (Berlin) 100, 558–559.

Gunasekaran, N., Long, L.E., Dawson, B.L., Hansen, G.H., Richardson, D.P., Li, K.M., Arnold, J.C., McGregor, I.S., 2009. Reintoxication: the release of fat-stored delta(9)-tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. Br. J. Pharmacol. 158, 1330–1337. https://doi.org/10.1111/j.1476-5381.2009.00399.x.

Hubner, C.B., Moreton, J.E., 1991. Effects of selective D1 and D2 dopamine antagonists on cocaine self-administration in the rat. Psychopharmacology (Berlin) 105, 151–156.

Hurd, Y.L., Yoon, M., Manini, A.F., Hernandez, S., Olmedo, R., Ostman, M., Jutras-Aswad, D., 2015. Early phase in the development of cannabidiol as a treatment for addiction: opioid relapse takes initial center stage. Neurotherapeutics 12, 807–815. https://doi.

org/10.1007/s13311-015-0373-7.
Kathmann, M., Flau, K., Redmer, A., Tränkle, C., Schlicker, E., 2006. Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 372, 354–361. https://doi.org/10.1007/s00210-006-0033-x.
Katsidoni, V., Anagnostou, I., Panagis, G., 2013. Cannabidiol inhibits the reward-facilitating effect of morphine: involvement of 5-HT1A receptors in the dorsal raphe nucleus. Addict. Biol. 18, 286–296. https://doi.org/10.1111/j.1369-1600.2012.00483.x.
Kelley, A.E., Baldo, B.A., Pratt, W.E., Will, M.J., 2005. Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward. Physiol. Behav. 86, 773–795. https://doi.org/10.1016/j.physbeh.2005.08.066.
Kirby, E.D., Kuwahara, A.A., Messer, R.L., Wyss-Coray, T., 2015. Adult hippocampal neural stem and progenitor cells regulate the neurogenic niche by secreting VEGF. Proc. Natl. Acad. Sci. U. S. A 112, 4128–4133. https://doi.org/10.1073/pnas.1422448112.
Laprairie, R.B., Bagher, A.M., Kelly, M.E.M., Denovan-Wright, E.M., 2015. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. Br. J. Pharmacol. 172, 4790–4805. https://doi.org/10.1111/bph.13250.
Lee, J.L.C., Bertoglio, L.J., Guimarães, F.S., Stevenson, C.W., 2017. Cannabidiol regulation of emotion and emotional memory processing: relevance for treating anxiety-related and substance abuse disorders. Br. J. Pharmacol. 174, 3242–3256. https://doi.org/10.1111/bph.13724.
Lin, S.J., Epps, S.A., West, C.H., Boss-Williams, K.A., Weiss, J.M., Weinshenker, D., 2012. Operant psychostimulant self-administration in a rat model of depression. Pharmacol. Biochem. Behav. 103, 380–385. https://doi.org/10.1016/j.pbb.2012.09.008.
Liou, G.I., Auchampach, J.A., Hillard, C.J., Zhu, G., Yousufzai, B., Mian, S., Khan, S., Khalifa, Y., 2008. Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A_{2A} adenosine receptor. Investig. Opthalmology Vis. Sci. 49, 5526. https://doi.org/10.1167/iovs.08-2196.
Loh, E.A., Fitch, T., Vickers, G., Roberts, D.C., 1992. Clozapine increases breaking points on a progressive-ratio schedule reinforced by intravenous cocaine. Pharmacol. Biochem. Behav. 42, 559–562.
López-Arnau, R., Luján, M.A., Duart-Castells, L., Pubill, D., Camarasa, J., Valverde, O., Escubedo, E., 2017. Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. Br. J. Pharmacol. 174, 1161–1173. https://doi.org/10.1111/bph.13771.
Loweth, J.A., Tseng, K.Y., Wolf, M.E., 2014. Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. Neuropharmacology 76, 287–300. https://doi.org/10.1016/j.neuropharm.2013.04.061.
Mahmud, A., Gallant, S., Sedki, F., D'Cunha, T., Shalev, U., 2017. Effects of an acute cannabidiol treatment on cocaine self-administration and cue-induced cocaine seeking in male rats. J. Psychopharmacol. 31, 96–104. https://doi.org/10.1177/0269881116667706.
Mallipeddi, S., Janero, D.R., Zvonok, N., Makriyannis, A., 2017. Functional selectivity at G-protein coupled receptors: advancing cannabinoid receptors as drug targets. Biochem. Pharmacol. 128, 1–11. https://doi.org/10.1016/j.bcp.2016.11.014.
Mariani, J.J., Pavlicova, M., Bisaga, A., Nunes, E.V., Brooks, D.J., Levin, F.R., 2012. Extended-release mixed amphetamine salts and topiramate for cocaine dependence: a randomized controlled trial. Biol. Psychiatry 72, 950–956. https://doi.org/10.1016/j.biopsych.2012.05.032.
Marinho, A.L.Z., Vila-Verde, C., Fogaça, M.V., Guimarães, F.S., 2015. Effects of intra-infralimbic prefrontal cortex injections of cannabidiol in the modulation of emotional behaviors in rats: contribution of 5HT1A receptors and stressful experiences. Behav. Brain Res. 286, 49–56. https://doi.org/10.1016/j.bbr.2015.02.023.
Markos, J.R., Harris, H.M., Gul, W., Elsohly, M.A., Sufka, K.J., 2017. Effects of cannabidiol on morphine conditioned place preference in mice. Planta Med. 84, 221–224. https://doi.org/10.1055/s-0043-117838.
Martínez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., Oyarzabal, J., Canela, E.I., Lanciego, J.L., Nadal, X., Navarro, G., Borea, P.A., Franco, R., 2017. Binding and signaling studies disclose a potential allosteric site for cannabidiol in cannabinoid CB2receptors. Front. Pharmacol. 8, 1–10. https://doi.org/10.3389/fphar.2017.00744.
McGregor, A., Lacosta, S., Roberts, D.C., 1993. L-tryptophan decreases the breaking point under a progressive ratio schedule of intravenous cocaine reinforcement in the rat. Pharmacol. Biochem. Behav. 44, 651–655.
McKerchar, T.L., Zarcone, T.J., Fowler, S.C., 2005. Differential acquisition of lever pressing in inbred and outbred mice: comparison of one-lever and two-lever procedures and correlation with differences in locomotor activity. J. Exp. Anal. Behav. 84, 339–356.
Moran, T.P., 2016. Anxiety and working memory capacity: a meta-analysis and narrative review. Psychol. Bull. 142, 831–864. https://doi.org/10.1037/bul0000051.
Moscoso-Castro, M., Gracia-Rubio, I., Ciruela, F., Valverde, O., 2016. Genetic blockade of adenosine A2A receptors induces cognitive impairments and anatomical changes related to psychotic symptoms in mice. Eur. Neuropsychopharmacol 1–14. https://doi.org/10.1016/j.euroneuro.2016.04.003.
Murphy, M., Mills, S., Winstone, J., Leishman, E., Wager-Miller, J., Bradshaw, H., Mackie, K., 2017. Chronic adolescent Δ 9 -tetrahydrocannabinol treatment of male mice leads to long-term cognitive and behavioral dysfunction, which are prevented by concurrent cannabidiol treatment. Cannabis Cannabinoid Res 2, 235–246. https://doi.org/10.1089/can.2017.0034.
Nazario, L.R., Antonioli, R., Capiotti, K.M., Hallak, J.E.C., Zuardi, A.W., Crippa, J.A.S., Bonan, C.D., da Silva, R.S., 2015. Caffeine protects against memory loss induced by high and non-anxiolytic dose of cannabidiol in adult zebrafish (Danio rerio). Pharmacol. Biochem. Behav. 135, 210–216. https://doi.org/10.1016/j.pbb.2015.06.008.
Negus, S.S., Henningfield, J., 2015. Agonist medications for the treatment of cocaine use disorder. Neuropsychopharmacology 40, 1815–1825. https://doi.org/10.1038/npp.2014.322.
Norris, C., Loureiro, M., Kramar, C., Zunder, J., Renard, J., Rushlow, W., Laviolette, S.R., 2016. Cannabidiol modulates fear memory formation through interactions with serotonergic transmission in the mesolimbic system. Neuropsychopharmacology 1–12. https://doi.org/10.1038/npp.2016.93.
Onaivi, E.S., Green, M.R., Martin, B.R., 1990. Pharmacological characterization of cannabinoids in the elevated plus maze. J. Pharmacol. Exp. Therapeut. 253, 1002–1009.
Ortega-Martínez, S., 2015. A new perspective on the role of the CREB family of transcription factors in memory consolidation via adult hippocampal neurogenesis. Front. Mol. Neurosci. 8, 46. https://doi.org/10.3389/fnmol.2015.00046.
Osborne, A.L., Solowij, N., Babic, I., Huang, X.-F., Weston-Green, K., 2017. Improved social interaction, recognition and working memory with cannabidiol treatment in a prenatal infection (poly I:C) rat model. Neuropsychopharmacology 42, 1447–1457. https://doi.org/10.1038/npp.2017.40.
Owens, M., Stevenson, J., Hadwin, J.A., Norgate, R., 2014. When does anxiety help or hinder cognitive test performance? The role of working memory capacity. Br. J. Psychol. 105, 92–101. https://doi.org/10.1111/bjop.12009.
Parker, L.A., Burton, P., Sorge, R.E., Yakiwchuk, C., Mechoulam, R., 2004. Effect of low doses of Δ9-tetrahydrocannabinol and cannabidiol on the extinction of cocaine-induced and amphetamine-induced conditioned place preference learning in rats. Psychopharmacology (Berlin) 175, 360–366. https://doi.org/10.1007/s00213-004-1825-7.
Pascoli, V., Terrier, J., Hiver, A., Lüscher, C., 2015. Sufficiency of mesolimbic dopamine neuron stimulation for the progression to addiction. Neuron 88, 1054–1066. https://doi.org/10.1016/j.neuron.2015.10.017.
Paxinos, G., Franklin, K., 2004. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Boston.
Pazos, M.R., Cinquina, V., Gómez, A., Layunta, R., Santos, M., Fernández-Ruiz, J., Martínez-Orgado, J., 2012. Cannabidiol administration after hypoxia–ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology 63, 776–783. https://doi.org/10.1016/j.neuropharm.2012.05.034.
Prenderville, J.A., Kelly, Á.M., Downer, E.J., 2015. The role of cannabinoids in adult neurogenesis. Br. J. Pharmacol. 172, 3950–3963. https://doi.org/10.1111/bph.13186.
Randall, P.A., Pardo, M., Nunes, E.J., López Cruz, L., Vemuri, V.K., Makriyannis, A., Baqi, Y., Müller, C.E., Correa, M., Salamone, J.D., 2012. Dopaminergic modulation of effort-related choice behavior as assessed by a progressive ratio chow feeding choice task: pharmacological studies and the role of individual differences. PloS One 7, e47934. https://doi.org/10.1371/journal.pone.0047934.
Ren, Y., Whittard, J., Higuera-Matas, A., Morris, C.V., Hurd, Y.L., 2009. Cannabidiol, a nonpsychotropic component of cannabis, inhibits cue-induced heroin seeking and normalizes discrete mesolimbic neuronal disturbances. J. Neurosci. 29, 14764–14769. https://doi.org/10.1523/JNEUROSCI.4291-09.2009.
Renard, J., Norris, C., Rushlow, W., Laviolette, S.R., 2016. Neuronal and molecular effects of cannabidiol on the mesolimbic dopamine system: implications for novel schizophrenia treatments. Neurosci. Biobehav. Rev. 75, 157–165. https://doi.org/10.1016/j.neubiorev.2017.02.006.
Richardson, N.R., Roberts, D.C.S., 1996. Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. J. Neurosci. Methods 66, 1–11. https://doi.org/10.1016/0165-0270(95)00153-0.
Robertson, D.C., Vickers, G., 1984. Atypical neuroleptics increase self-administration of cocaine: an evaluation of a behavioural screen for antipsychotic activity. Psychopharmacology (Berlin) 82, 135–139.
Roberts, D.C.S., Gabriele, A., Zimmer, B.A., 2013. Conflation of cocaine seeking and cocaine taking responses in IV self-administration experiments in rats: methodological and interpretational considerations. Neurosci. Biobehav. Rev. 37, 2026–2036. https://doi.org/10.1016/j.neubiorev.2013.04.017.
Russo, E.B., Burnett, A., Hall, B., Parker, K.K., 2005. Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem. Res. 30, 1037–1043. https://doi.org/10.1007/s11064-005-6978-1.
Schiavon, A.P., Bonato, J.M., Milani, H., Guimarães, F.S., Weffort de Oliveira, R.M., 2016. Influence of single and repeated cannabidiol administration on emotional behavior and markers of cell proliferation and neurogenesis in non-stressed mice. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 64, 27–34. https://doi.org/10.1016/j.pnpbp.2015.06.017.
Snyder, J.S., Choe, J.S., Clifford, M.A., Jeurling, S.I., Hurley, P., Brown, A., Kamhi, J.F., Cameron, H.A., 2009. Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. J. Neurosci. 29, 14484–14495. https://doi.org/10.1523/JNEUROSCI.1768-09.2009.
Soria, G., Barbano, M.F., Maldonado, R., Valverde, O., 2008. A reliable method to study cue-, priming-, and stress-induced reinstatement of cocaine self-administration in mice. Psychopharmacology (Berlin) 199, 593–603. https://doi.org/10.1007/s00213-008-1184-x.
Tourino, C., Valjent, E., Ruiz-Medina, J., Herve, D., Ledent, C., Valverde, O., 2012. The orphan receptor GPR3 modulates the early phases of cocaine reinforcement. Br. J. Pharmacol. 167, 892–904. https://doi.org/10.1111/j.1476-5381.2012.02043.x.
Viudez-Martínez, A., García-Gutiérrez, M.S., Navarrón, C.M., Morales-Calero, M.I., Navarrete, F., Torres-Suárez, A.I., Manzanares, J., 2017. Cannabidiol reduces ethanol consumption, motivation and relapse in mice. Addict. Biol. 23, 154–164. https://doi.org/10.1111/adb.12495.
Volkow, N.D., Koob, G.F., McLellan, A.T., 2016. Neurobiologic advances from the brain disease model of addiction. N. Engl. J. Med. 374, 363–371. https://doi.org/10.1056/NEJMra1511480.

Ware, M.A., 2018. Medical cannabis research: issues and priorities. Neuropsychopharmacology 43, 214–215. https://doi.org/10.1038/npp.2017.222.
Wenzel, J.M., Cheer, J.F., 2018. Endocannabinoid regulation of reward and reinforcement through interaction with dopamine and endogenous opioid signaling. Neuropsychopharmacology 43, 103–115. https://doi.org/10.1038/npp.2017.126.
Wolf, M.E., 2016. Synaptic mechanisms underlying persistent cocaine craving. Nat. Rev. Neurosci. 17, 351–365. https://doi.org/10.1038/nrn.2016.39.
Wolf, S.A., Bick-Sander, A., Fabel, K., Leal-Galicia, P., Tauber, S., Ramirez-Rodriguez, G., Müller, A., Melnik, A., Waltinger, T.P., Ullrich, O., Kempermann, G., 2010. Cannabinoid receptor CB1 mediates baseline and activity-induced survival of new neurons in adult hippocampal neurogenesis. Cell Commun. Signal. 8, 12. https://doi.org/10.1186/1478-811X-8-12.
Wu, X., French, E.D., 2000. Effects of chronic delta9-tetrahydrocannabinol on rat midbrain dopamine neurons: an electrophysiological assessment. Neuropharmacology 39, 391–398.
Yokel, R.A., Wise, R.A., 1975. Increased lever pressing for amphetamine after pimozide in rats: implications for a dopamine theory of reward. Science 187, 547–549.
Zhang, J., Cai, C.Y., Wu, H.Y., Zhu, L.J., Luo, C.X., Zhu, D.Y., 2016. CREB-mediated synaptogenesis and neurogenesis is crucial for the role of 5-HT1a receptors in modulating anxiety behaviors. Sci. Rep. 6. https://doi.org/10.1038/srep29551.
Zuardi, A.W., Rodrigues, N.P., Silva, A.L., Bernardo, S.A., Hallak, J.E.C., Guimarães, F.S., Crippa, J.A.S., 2017. Inverted U-shaped dose-response curve of the anxiolytic effect of cannabidiol during public speaking in real life. Front. Pharmacol. 8, 259. https://doi.org/10.3389/fphar.2017.00259.

Annex 2

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

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Neuropharmacology 146: 50-64 (2019)

DOI: 10.1016/j.neuropharm.2018.11.011

Neuropharmacology 146 (2019) 50–64

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

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:
Social defeat
Oxytocin
Cocaine
BDNF
Self-administration
Conditioned place preference

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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https://doi.org/10.1016/j.neuropharm.2018.11.011
Received 27 July 2018; Received in revised form 8 November 2018; Accepted 9 November 2018
Available online 16 November 2018
0028-3908/

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		Social defeat / Exploration				
Brain samples		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 ($\chi2 = 6.667$; $p = 0.01$) (9 sessions) or OXT-EXP ($\chi2 = 6.418$; $p = 0.011$) (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

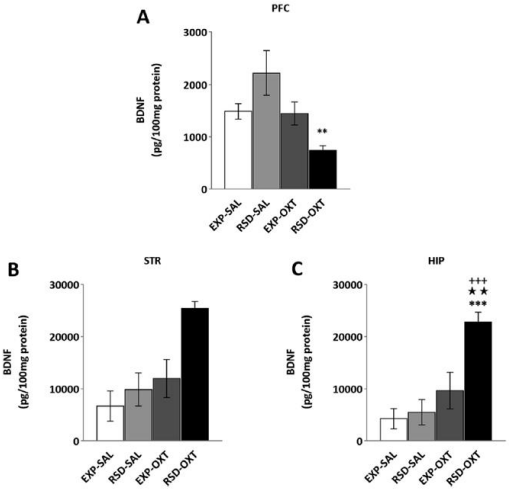

Fig. 1. Effects of RSD and/or OXT treatment on BDNF levels. Mean of BDNF levels (pg/100 mg protein) in (A) PFC, (B) STR and (C) HIP. Two-way ANOVA and subsequent Bonferroni post-hoc test. ★★$p < 0.01$, versus EXP-OXT group. **$p < 0.01$, ***$p < 0.001$, versus RSD-SAL group. +$p < 0.001$ versus EXP-SAL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

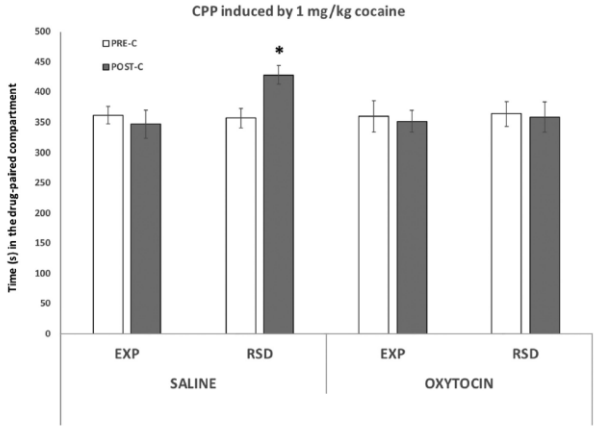

Fig. 2. Administration of oxytocin before each social defeat episode blocked acquisition of the CPP induced by 1 mg/kg of cocaine in defeated mice. Before the social stress protocol animals were randomly assigned to the following groups according to treatment: saline (SAL-EXP n = 14; SAL-RSD n = 15); 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.

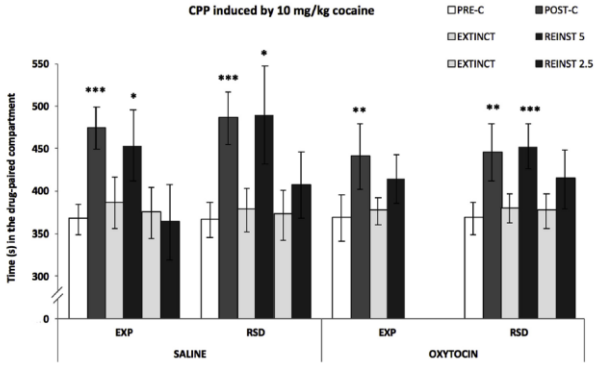

Fig. 3. Administration of oxytocin before each social defeat does not affect acquisition of the CPP induced by 10 mg/kg of cocaine. Before the social stress protocol animals were randomly assigned to one of the following groups according to the pre-treatment they received: saline (SAL-EXP n = 14; SAL-RSD 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.

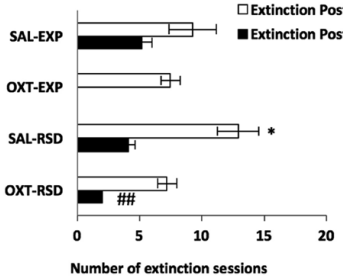

Fig. 4. Effect of oxytocin administration before each social defeat on the number of sessions needed to extinguish the preference induced by 10 mg/kg of cocaine. White bars represent the preference induced by 10 mg/kg cocaine while grey bars represent the preference reinstated with a priming dose of 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-

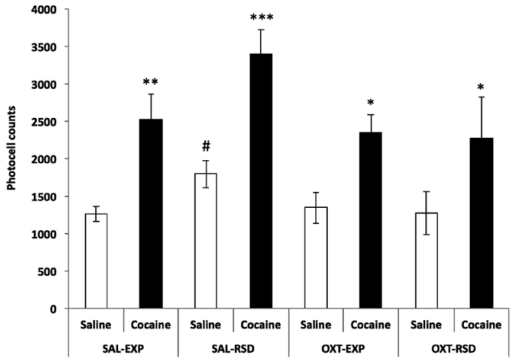

Fig. 5. Administration of 1 mg/kg of oxytocin before each social defeat blocks the increased locomotor sensitization to cocaine induced by social defeat stress. During the induction phase, half of the animals in each group received pretreatment with saline (S) (S-SAL-EXP n = 8; S-SAL-RSD n = 8; S-OXT-EXP 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.

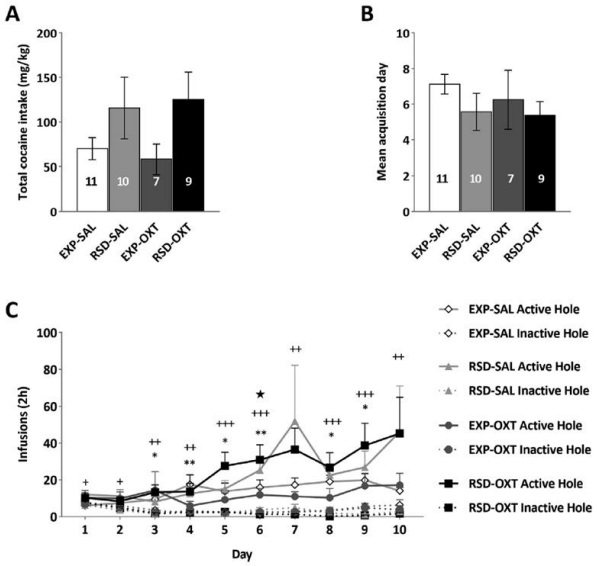

Fig. 6. Effects of RSD and/or OXT treatment on acquisition of cocaine self-administration behavior. (A) Mean of total cocaine intake (mg cocaine/kg mice) throughout the 10 days of the acquisition phase (2 h per session). (B) Mean of days needed to acquire self-administration behavior. (C) Mean number 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-

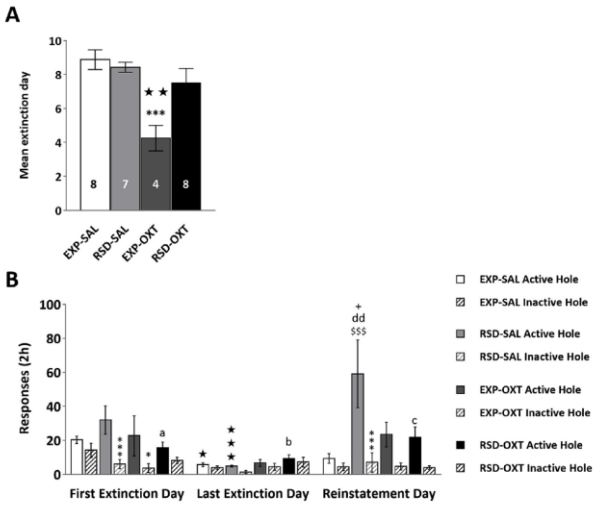

Fig. 7. Effects of RSD and/or OXT treatment on the extinction and reinstatement of cocaine self-administration behavior. (A) Mean of days needed to extinguish cocaine seeking-behavior. Two-way ANOVA and Bonferroni's post hoc test. ***p < 0.001, effect of Treatment in EXP mice. ★★p < 0.01, effect of Stress 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.

Funding sources

This work was supported by the Ministerio de Economía y Competitividad, Dirección General de Investigación, [grant numbers PSI2014-51847-R and PSI 2017-83023-R]; Spanish Ministry of Economy, Innovation and Competiveness (SAF2016-75347-R). Instituto de Salud Carlos III, Red de Trastornos Adictivos (RTA) [grant numbers RETICS RD06/0001/1006; RD12/0028/0005; and RD/16/0017/0010] and Unión Europea, Fondos FEDER "A way to build Europe". The Department of Experimental and Health Sciences (UPF) is an "Unidad de Excelencia María de Maeztu" funded by the MINECO (Ref. MDM-2014-0370). A.C-Z received CONACYT grant from the Government of Mexico. M.A.L. and C.F.P received FPU grant from the Spanish Ministry of Economy, Innovation and Competiveness (15/02492 and 14/02279). The authors declare no conflicts of interest.

Acknowledgements

We wish to thank Brian Normanly for his English language editing.

References

Aguilar, M.A., Rodríguez-Arias, M., Miñarro, J., 2009. Neurobiological mechanisms of the reinstatement of drug-conditioned place preference. Brain Res. Rev. 59, 253–277. https://doi.org/10.1016/j.brainresrev.2008.08.002.

Albrechet-Souza, L., Viola, T.W., Grassi-Oliveira, R., Miczek, K.A., de Almeida, R.M.M., 2017. Corticotropin releasing factor in the bed nucleus of the stria terminalis in socially defeated and non-stressed mice with a history of chronic alcohol intake. Front. Pharmacol. 8, 762. https://doi.org/10.3389/fphar.2017.00762.

Arenas, M.C., Daza-Losada, M., Vidal-Infer, A., Aguilar, M.A., Miñarro, J., Rodríguez-Arias, M., 2014. Capacity of novelty-induced locomotor activity and the hole-board test to predict sensitivity to the conditioned rewarding effects of cocaine. Physiol. Behav. 133, 152–160. https://doi.org/10.1016/j.physbeh.2014.05.028.

Balmus, I.M., Lefter, R., Ciobica, A., Antioch, I., Ababei, D., Dobrin, R., 2018. Preliminary data on some behavioral changes induced by short-term intraperitoneal oxytocin administration in aged rats. Psychiatr. Danub. 30, 91–98. https://doi.org/10.24869/psyd.2018.91.

Baracz, S.J., Cornish, J.L., 2016. The neurocircuitry involved in oxytocin modulation of methamphetamine addiction. Front. Neuroendocrinol. 43, 1–18. https://doi.org/10.1016/j.yfrne.2016.08.001.

Baracz, S.J., Everett, N.A., Cornish, J.L., 2018. The impact of early life stress on the central oxytocin system and susceptibility for drug addiction: applicability of oxytocin as a pharmacotherapy. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2018.08.014.

Berhow, M.T., Russell, D.S., Terwilliger, R.Z., Beitner-Johnson, D., Self, D.W., Lindsay, R.M., Nestler, E.J., 1995. Influence of neurotrophic factors on morphine- and cocaine-induced biochemical changes in the mesolimbic dopamine system. Neuroscience 68, 969–979. https://doi.org/10.1016/0306-4522(95)00207-Y.

Berton, O., McClung, C.A., DiLeone, R.J., Krishnan, V., Renthal, W., Russo, S.J., Graham, D., Tsankova, N.M., Bolanos, C.A., Rios, M., Monteggia, L.M., Self, D.W., Nestler, E.J., 2006. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311, 864–868. https://doi.org/10.1126/science.1120972.

Björkholm, C., Monteggia, L.M., 2016. BDNF - a key transducer of antidepressant effects. Neuropharmacology 102, 72–79. https://doi.org/10.1016/j.neuropharm.2015.10.034.

Boccia, M.M., Baratti, C.M., 2000. Involvement of central cholinergic mechanisms in the effects of oxytocin and an oxytocin receptor antagonist on retention performance in mice. Neurobiol. Learn. Mem. 74, 217–228. https://doi.org/10.1006/nlme.1999.3954.

Boccia, M.M., Kopf, S.R., Baratti, C.M., 1998. Effects of a single administration of oxytocin or vasopressin and their interactions with two selective receptor antagonists on memory storage in mice. Neurobiol. Learn. Mem. 69, 136–146. https://doi.org/10.1006/nlme.1997.3817.

Boyson, C.O., Holly, E.N., Shimamoto, A., Albrechet-Souza, L., Weiner, L.A., DeBold, J.F., Miczek, K.A., 2014. Social stress and CRF-dopamine interactions in the VTA: role in long-term escalation of cocaine self-administration. J. Neurosci. 34, 6659–6667. https://doi.org/10.1523/JNEUROSCI.3942-13.2014.

Boyson, C.O., Miguel, T.T., Quadros, I.M., DeBold, J.F., Miczek, K.A., 2011. Prevention of social stress-escalated cocaine self-administration by CRF-R1 antagonist in the rat VTA. Psychopharmacology 218, 257–269. https://doi.org/10.1007/s00213-011-2266-8.

Burke, A.R., Miczek, K.A., 2015. Escalation of cocaine self-administration in adulthood after social defeat of adolescent rats: role of social experience and adaptive coping behavior. Psychopharmacology 232, 3067–3079. https://doi.org/10.1007/s00213-.

Carson, D.S., Cornish, J.L., Guastella, A.J., Hunt, G.E., McGregor, I.S., 2010. Oxytocin decreases methamphetamine self-administration, methamphetamine hyperactivity, and relapse to methamphetamine-seeking behaviour in rats. Neuropharmacology 58, 38–43. https://doi.org/10.1016/j.neuropharm.2009.06.018.

Chini, B., Leonzino, M., Braida, D., Sala, M., 2014. Learning about oxytocin:

pharmacologic and behavioral issues. Biol. Psychiatry 76, 360–366. https://doi.org/10.1016/j.biopsych.2013.08.029.
Cohen, H., Kaplan, Z., Kozlovsky, N., Gidron, Y., Matar, M.A., Zohar, J., 2010. Hippocampal microinfusion of oxytocin attenuates the behavioural response to stress by means of dynamic interplay with the glucocorticoid-catecholamine responses. J. Neuroendocrinol. 22, 889–904. https://doi.org/10.1111/j.1365-2826.2010.02003.x.
Coppens, C.M., Siripornmongcolchai, T., Wibrand, K., Alme, M.N., Buwalda, B., de Boer, S.F., Koolhaas, J.M., Bramham, C.R., 2011. Social defeat during adolescence and adulthood differentially induce BDNF-regulated immediate early genes. Front. Behav. Neurosci. 5, 72. https://doi.org/10.3389/fnbeh.2011.00072.
Covington, H.E., Kikusui, T., Goodhue, J., Nikulina, E.M., Hammer, R.P., Miczek, K.A., 2005. Brief social defeat stress: long lasting effects on cocaine taking during a binge and zif268 mRNA expression in the amygdala and prefrontal cortex. Neuropsychopharmacology 30, 310–321. https://doi.org/10.1038/sj.npp.1300587.
Covington, H.E., Miczek, K.A., 2001. Repeated social-defeat stress, cocaine or morphine: effects on behavioral sensitization and intravenous cocaine self-administration "binges. Psychopharmacology 158, 388–398. https://doi.org/10.1007/s002130100858.
Cox, B.M., Young, A.B., See, R.E., Reichel, C.M., 2013. Sex differences in methamphetamine seeking in rats: impact of oxytocin. Psychoneuroendocrinology 38, 2343–2353. https://doi.org/10.1016/j.psyneuen.2013.05.005.
Dayi, A., Cetin, F., Sisman, A.R., Aksu, I., Tas, A., Gönenc, S., Uysal, N., 2015. The effects of oxytocin on cognitive defect caused by chronic restraint stress applied to adolescent rats and on hippocampal VEGF and BDNF levels. Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 21, 69–75. https://doi.org/10.12659/MSM.893159.
Daza-Losada, M., Rodríguez-Arias, M., Maldonado, C., Aguilar, M.A., Guerri, C., Miñarro, J., 2009. Acute behavioural and neurotoxic effects of MDMA plus cocaine in adolescent mice. Neurotoxicol. Teratol. 31, 49–59. https://doi.org/10.1016/j.ntt.2008.07.005.
Duclot, F., Kabbaj, M., 2013. Individual differences in novelty seeking predict subsequent vulnerability to social defeat through a differential epigenetic regulation of brain-derived neurotrophic factor expression. J. Neurosci. 33, 11048–11060. https://doi.org/10.1523/JNEUROSCI.0199-13.2013.
Duque-Wilckens, N., Steinman, M.Q., Busnelli, M., Chini, B., Yokoyama, S., Pham, M., Laredo, S.A., Hao, R., Perkeybile, A.M., Minie, V.A., Tan, P.B., Bales, K.L., Trainor, B.C., 2018. Oxytocin receptors in the anteromedial bed nucleus of the stria terminalis promote stress-induced social avoidance in female California mice. Biol. Psychiatry 83, 203–213. https://doi.org/10.1016/j.biopsych.2017.08.024.
Ebner, K., Wotjak, C.T., Landgraf, R., Engelmann, M., 2000. A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. Brain Res. 872, 87–92. https://doi.org/10.1016/S0006-8993(00)02464-1.
Eckstein, M., Scheele, D., Weber, K., Stoffel-Wagner, B., Maier, W., Hurlemann, R., 2014. Oxytocin facilitates the sensation of social stress. Hum. Brain Mapp. 35, 4741–4750. https://doi.org/10.1002/hbm.22508.
Engelmann, M., Ebner, K., Landgraf, R., Holsboer, F., Wotjak, C.T., 1999. Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. J. Neuroendocrinol. 11, 867–872. https://doi.org/10.1046/j.1365-2826.1999.00403.x.
Fanous, S., Hammer, R.P., Nikulina, E.M., 2010. Short- and long-term effects of intermittent social defeat stress on brain-derived neurotrophic factor expression in mesocorticolimbic brain regions. Neuroscience 167, 598–607. https://doi.org/10.1016/j.neuroscience.2010.02.064.
Ferrer-Pérez, C., Reguilón, M.D., Manzanedo, C., Aguilar, M.A., Miñarro, J., Rodríguez-Arias, M., 2018. Antagonism of corticotropin-releasing factor CRF1receptors blocks the enhanced response to cocaine after social stress. Eur. J. Pharmacol. 823, 87–95. https://doi.org/10.1016/j.ejphar.2018.01.052.
Flanagin, B.A., Cook, E.H., De Wit, H., 2006. An association study of the brain-derived neurotrophic factor Val66Met polymorphism and amphetamine response. Am. J. Med. Genet. Part B Neuropsychiatr. Genet. 141, 576–583. https://doi.org/10.1002/ajmg.b.30327.
Fumagalli, F., Di Pasquale, L., Caffino, L., Racagni, G., Riva, M.A., 2007. Repeated exposure to cocaine differently modulates BDNF mRNA and protein levels in rat striatum and prefrontal cortex. Eur. J. Neurosci. 26, 2756–2763. https://doi.org/10.1111/j.1460-9568.2007.05918.x.
Gaffori, O.J.W., De Wied, D., 1988. Bimodal effect of oxytocin on avoidance behavior may be caused by the presence of two peptide sequences with opposite action in the same molecule. Eur. J. Pharmacol. 147, 157–162. https://doi.org/10.1016/0014-2999(88)90774-1.
Gard, P.R., Naylor, C., Ali, S., Partington, C., 2012. Blockade of pro-cognitive effects of angiotensin IV and physostigmine in mice by oxytocin antagonism. Eur. J. Pharmacol. 683, 155–160. https://doi.org/10.1016/j.ejphar.2012.02.048.
Gimpl, G., Fahrenholz, F., 2001. The oxytocin receptor system: structure, function, and regulation. Physiol. 81, 629–683. https://doi.org/10.1152/physrev.2001.81.2.629.
Grillon, C., Krimsky, M., Charney, D.R., Vytal, K., Ernst, M., Cornwell, B., 2013. Oxytocin increases anxiety to unpredictable threat. Mol. Psychiatr. 18, 958–960. https://doi.org/10.1038/mp.2012.156.
Georgiou, P., Zanos, P., Hourani, S., Kitchen, I., Bailey, A., 2016. Cocaine abstinence induces emotional impairment and brain region-specific upregulation of the oxytocin receptor binding. Eur. J. Neurosci. 44, 2446–2454. https://doi.org/10.1111/ejn.13348.
Grippo, A.J., Pournajafi-Nazarloo, H., Sanzenbacher, L., Trahanas, D.M., McNeal, N., Clarke, D.A., Porges, S.W., Sue Carter, C., 2012. Peripheral oxytocin administration buffers autonomic but not behavioral responses to environmental stressors in isolated prairie voles. Stress 15, 149–161. https://doi.org/10.3109/10253890.2011.605486.
Guillin, O., Diaz, J., Carroll, P., Griffon, N., Schwartz, J.C., Sokoloff, P., 2001. BDNF controls dopamine D3receptor expression and triggers behavioural sensitization. Nature 411, 86–89. https://doi.org/10.1038/35075076.
Hall, F.S., Drgonova, J., Goeb, M., Uhl, G.R., 2003. Reduced behavioral effects of cocaine in heterozygous brain-derived neurotrophic factor (BDNF) knockout mice. Neuropsychopharmacology 28, 1485–1490. https://doi.org/10.1038/sj.npp.1300192.
Hammels, C., Pishva, E., De Vry, J., van den Hove, D.L.A., Prickaerts, J., van Winkel, R., Selten, J.P., Lesch, K.P., Daskalakis, N.P., Steinbusch, H.W.M., van Os, J., Kenis, G., Rutten, B.P.F., 2015. Defeat stress in rodents: from behavior to molecules. Neurosci. Biobehav. Rev. 59, 111–140. https://doi.org/10.1016/j.neubiorev.2015.10.006.
Han, X., DeBold, J.F., Miczek, K.A., 2017. Prevention and reversal of social stress-escalated cocaine self-administration in mice by intra-VTA CRFR1 antagonism. Psychopharmacology 234, 2813–2821. https://doi.org/10.1007/s00213-017-4676-8.
Havranek, T., Zatkova, M., Lestanova, Z., Bacova, Z., Mravec, B., Hodosy, J., Strbak, V., Bakos, J., 2015. Intracerebroventricular oxytocin administration in rats enhances object recognition and increases expression of neurotrophins, microtubule-associated protein 2, and synapsin I. J. Neurosci. Res. 93, 893–901. https://doi.org/10.1002/jnr.23559.
Heinrichs, M., Domes, G., 2008. Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. Prog. Brain Res. 170, 337–350. https://doi.org/10.1016/S0079-6123(08)00428-7.
Holly, E.N., Boyson, C.O., Montagud-Romero, S., Stein, D.J., Gobrogge, K.L., DeBold, J.F., Miczek, K.A., 2016. Episodic social stress-escalated cocaine self-administration: role of phasic and tonic corticotropin releasing factor in the anterior and posterior ventral tegmental area. J. Neurosci. 36, 4093–4105. https://doi.org/10.1523/JNEUROSCI.2232-15.2016.
Hyman, S.E., Malenka, R.C., Nestler, E.J., 2006. NEURAL MECHANISMS OF ADDICTION: the role of reward-related learning and memory. Annu. Rev. Neurosci. 29, 565–598. https://doi.org/10.1146/annurev.neuro.29.051605.113009.
Hymel, K.A., Eans, S.O., Sitchenko, K.L., Gomes, S.M., Lukowsky, A.L., Medina, J.M., Sypek, E.I., Carey, A.N., McLaughlin, J.P., 2014. Stress-induced increases in depression-like and cocaine place-conditioned behaviors are reversed by disruption of memories during reconsolidation. Behav. Pharmacol. 25, 599–608. https://doi.org/10.1097/FBP.0000000000000074.
Johnson, Z.V., Young, L.J., 2017. Oxytocin and vasopressin neural networks: implications for social behavioral diversity and translational neuroscience. Neurosci. Biobehav. Rev. 76, 87–98. https://doi.org/10.1016/j.neubiorev.2017.01.034.
Kalivas, P.W., Pierce, R.C., Cornish, J., Sorg, B.A., 1998. A role for sensitization in craving and relapse in cocaine addiction. J. Psychopharmacol. 12, 49–53. https://doi.org/10.1177/026988119801200107.
Kalivas, P.W., Stewart, J., 1991. Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. Brain Res. Rev. 16, 223–244. https://doi.org/10.1016/0165-0173(91)90007-U.
King, C.E., Griffin, W.C., Luderman, L.N., Kates, M.M., McGinty, J.F., Becker, H.C., 2017. Oxytocin reduces ethanol self-administration in mice. Alcohol Clin. Exp. Res. 41, 955–964. https://doi.org/10.1111/acer.13359.
Kirsch, P., 2005. Oxytocin modulates neural circuitry for social cognition and fear in humans. J. Neurosci. 25, 11489–11493. https://doi.org/10.1523/JNEUROSCI.3984-05.2005.
Kovács, G.L., Bohus, B., Versteeg, D.H.G., Ronald De Kloet, E., De Wied, D., 1979. Effect of oxytocin and vasopressin on memory consolidation: sites of action and catecholaminergic correlates after local microinjection into limbic-midbrain structures. Brain Res. 175, 303–314. https://doi.org/10.1016/0006-8993(79)91009-6.
Kovács, G.L., De Wied, D., 1994. Peptidergic modulation of learning and memory processes. Pharmacol. Rev. 46, 269–291.
Kovàcs, G.L., Faludi, M., Falkay, G., Telegdy, G., 1986. Peripheral oxytocin treatment modulates central dopamine transmission in the mouse limbic structures. Neurochem. Int. 9, 481–485. https://doi.org/10.1016/0197-0186(86)90138-5.
Kovàcs, G.L., Sarnyai, Z., Babarczi, E., Szabó, G., Telegdy, G., 1990. The role of oxytocin-dopamine interactions in cocaine-induced locomotor hyperactivity. Neuropharmacology 29, 365–368. https://doi.org/10.1016/0028-3908(90)90095-9.
Krishnan, V., Han, M.H., Graham, D.L., Berton, O., Renthal, W., Russo, S.J., LaPlant, Q., Graham, A., Lutter, M., Lagace, D.C., Ghose, S., Reister, R., Tannous, P., Green, T.A., Neve, R.L., Chakravarty, S., Kumar, A., Eisch, A.J., Self, D.W., Lee, F.S., Tamminga, C.A., Cooper, D.C., Gershenfeld, H.K., Nestler, E.J., 2007. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131, 391–404. https://doi.org/10.1016/j.cell.2007.09.018.
Lagace, D.C., Donovan, M.H., DeCarolis, N.A., Farnbauch, L.A., Malhotra, S., Berton, O., Nestler, E.J., Krishnan, V., Eisch, A.J., 2010. Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. Proc. Natl. Acad. Sci. Unit. States Am. 107, 4436–4441. https://doi.org/10.1073/pnas.0910072107.
Land, B.B., Bruchas, M.R., Schattauer, S., Giardino, W.J., Aita, M., Messinger, D., Hnasko, T.S., Palmiter, R.D., Chavkin, C., 2009. Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. Proc. Natl. Acad. Sci. Unit. States Am. 106, 19168–19173. https://doi.org/10.1073/pnas.0910705106.
Laviola, G., Zoratto, F., Ingiosi, D., Carito, V., Huzard, D., Fiore, M., Macrì, S., 2017. Low empathy-like behaviour in male mice associates with impaired sociability, emotional memory, physiological stress reactivity and variations in neurobiological regulations. PLoS One 12. https://doi.org/10.1371/journal.pone.0188907.
Le Foll, B., Diaz, J., Sokoloff, P., 2005. A single cocaine exposure increases BDNF and D3receptor expression: implications for drug-conditioning. Neuroreport 16, 175–178. https://doi.org/10.1097/00001756-200502080-00022.
Leonard, M.Z., DeBold, J.F., Miczek, K.A., 2017. Escalated cocaine "binges" in rats: enduring effects of social defeat stress or intra-VTA CRF. Psychopharmacology 234, 2823–2836. https://doi.org/10.1007/s00213-017-4677-7.

Leong, K.C., Freeman, L.R., Berini, C.R., Ghee, S.M., See, R.E., Reichel, C.M., 2017. Oxytocin reduces cocaine cued fos activation in a regionally specific manner. Int. J. Neuropsychopharmacol. 20, 844–854. https://doi.org/10.1093/ijnp/pyx058.

Leong, K.C., Zhou, L., Ghee, S.M., See, R.E., Reichel, C.M., 2016. Oxytocin decreases cocaine taking, cocaine seeking, and locomotor activity in female rats. Exp. Clin. Psychopharmacol 24, 55–64. https://doi.org/10.1037/pha0000058.

Leuner, B., Caponiti, J.M., Gould, E., 2011. Oxytocin stimulates adult neurogenesis even under conditions of stress and elevated glucocorticoids. Hippocampus 22, 861–868. https://doi.org/10.1002/hipo.20947.

Light, K.C., Grewen, K.M., Amico, J.A., Boccia, M., Brownley, K.A., Johns, J.M., 2004. Deficits in plasma oxytocin responses and increased negative affect, stress, and blood pressure in mothers with cocaine exposure during pregnancy. Addict. Behav. 29, 1541–1564. https://doi.org/10.1016/j.addbeh.2004.02.062.

Litvin, Y., Murakami, G., Pfaff, D.W., 2011. Effects of chronic social defeat on behavioral and neural correlates of sociality: vasopressin, oxytocin and the vasopressinergic V1b receptor. Physiol. Behav. 103, 393–403. https://doi.org/10.1016/j.physbeh.2011.03.007.

Logrip, M.L., Zorrilla, E.P., Koob, G.F., 2012. Stress modulation of drug self-administration: implications for addiction comorbidity with post-traumatic stress disorder. Neuropharmacology 62, 552–564. https://doi.org/10.1016/j.neuropharm.2011.07.007.

López-Arnau, R., Luján, M.A., Duart-Castells, L., Pubill, D., Camarasa, J., Valverde, O., Escubedo, E., 2017. Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. Br. J. Pharmacol. 174, 1161–1173. https://doi.org/10.1111/bph.13771.

Lu, L., Liu, D., Ceng, X., 2001. Corticotropin-releasing factor receptor type 1 mediates stress-induced relapse to cocaine-conditioned place preference in rats. Eur. J. Pharmacol. 415, 203–208. https://doi.org/10.1016/S0014-2999(01)00840-8.

Lukas, M., Neumann, I.D., 2014. Social preference and maternal defeat-induced social avoidance in virgin female rats: sex differences in involvement of brain oxytocin and vasopressin. J. Neurosci. Methods 234, 101–107. https://doi.org/10.1016/j.jneumeth.2014.03.013.

Lukas, M., Toth, I., Reber, S.O., Slattery, D.A., Veenema, A.H., Neumann, I.D., 2011. The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. Neuropsychopharmacology 36, 2159–2168. https://doi.org/10.1038/npp.2011.95.

Lumley, L.A., Charles, R.F., Charles, R.C., Hebert, M.A., Morton, D.M., Meyerhoff, J.L., 2000. Effects of social defeat and of diazepam on behavior in a resident-intruder test in male DBA/2 mice. Pharmacol. Biochem. Behav. 67, 433–447. https://doi.org/10.1016/S0091-3057(00)00382-8.

Macedo, G.C., Morita, G.M., Domingues, L.P., Favoretto, C.A., Suchecki, D., Quadros, I.M.H., 2018. Consequences of continuous social defeat stress on anxiety- and depressive-like behaviors and ethanol reward in mice. Horm. Behav. 97, 154–161. https://doi.org/10.1016/j.yhbeh.2017.10.007.

MacFadyen, K., Loveless, R., Delucca, B., Wardley, K., Deogan, S., Thomas, C., Peris, J., 2016. Peripheral oxytocin administration reduces ethanol consumption in rats. Pharmacol. Biochem. Behav. 140, 27–32. https://doi.org/10.1016/j.pbb.2015.10.014.

Maldonado, C., Rodríguez-Arias, M., Castillo, A., Aguilar, M.A., Miñarro, J., 2006. Gamma-hydroxybutyric acid affects the acquisition and reinstatement of cocaine-induced conditioned place preference in mice. Behav. Pharmacol. 17, 119–131. https://doi.org/10.1097/01.fbp.0000190685.84984.ec.

Malenka, R.C., Bear, M.F., 2004. LTP and LTD: an embarrassment of riches. Neuron 44, 5–21. https://doi.org/10.1016/j.neuron.2004.09.012.

McGough, N.N.H., 2004. RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J. Neurosci. 24, 10542–10552. https://doi.org/10.1523/JNEUROSCI.3714-04.2004.

McLaughlin, J.P., Li, S., Valdez, J., Chavkin, T.A., Chavkin, C., 2006. Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system. Neuropsychopharmacology 31, 1241–1248. https://doi.org/10.1038/sj.npp.1300872.

McRae-Clark, A.L., Baker, N.L., Maria, M.M.S., Brady, K.T., 2013. Effect of oxytocin on craving and stress response in marijuana-dependent individuals: a pilot study. Psychopharmacology 228, 623–631. https://doi.org/10.1007/s00213-013-3062-4.

Meerlo, P., Koehl, M., Van Der Borght, K., Turek, F.W., 2002. Sleep restriction alters the hypothalamic-pituitary-adrenal response to stress. J. Neuroendocrinol. 14 (5), 397–402. https://doi.org/10.1046/j.0007-1331.2002.00790.x.

Meredith, G.E., Callen, S., Scheuer, D.A., 2002. Brain-derived neurotrophic factor expression is increased in the rat amygdala, piriform cortex and hypothalamus following repeated amphetamine administration. Brain Res. 949, 218–227. https://doi.org/10.1016/S0006-8993(02)03160-8.

Miczek, K.A., Nikulina, E.M., Shimamoto, A., Covington, H.E., 2011. Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. J. Neurosci. 31, 9848–9857. https://doi.org/10.1523/JNEUROSCI.0637-11.2011.

Miczek, K.A., Thompson, M.L., Shuster, L., 1982. Opioid-like analgesia in defeated mice. Science 215, 1520–1522. https://doi.org/10.1126/science.7199758.

Montagud-Romero, S., Daza-Losada, M., Vidal-Infer, A., Maldonado, C., Aguilar, M.A., Miñarro, J., Rodríguez-Arias, M., 2014. The novelty-seeking phenotype modulates the long-lasting effects of intermittent ethanol administration during adolescence. PLoS One 9. https://doi.org/10.1371/journal.pone.0092576.

Montagud-Romero, S., Montesinos, J., Pascual, M., Aguilar, M.A., Roger-Sanchez, C., Guerri, C., Miñarro, J., Rodríguez-Arias, M., 2016a. Up-regulation of histone acetylation induced by social defeat mediates the conditioned rewarding effects of cocaine. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 70, 39–48. https://doi.org/10.1016/j.pnpbp.2016.04.016.

Montagud-Romero, S., Nuñez, C., Blanco-Gandia, M.C., Martínez-Laorden, E., Aguilar, M.A., Navarro-Zaragoza, J., Almela, P., Milanés, M.V., Laorden, M.L., Miñarro, J., Rodríguez-Arias, M., 2017. Repeated social defeat and the rewarding effects of cocaine in adult and adolescent mice: dopamine transcription factors, proBDNF signaling pathways, and the TrkB receptor in the mesolimbic system. Psychopharmacology 234, 2063–2075. https://doi.org/10.1007/s00213-017-4612-y.

Montagud-Romero, S., Reguilon, M.D., Roger-Sanchez, C., Pascual, M., Aguilar, M.A., Guerri, C., Miñarro, J., Rodríguez-Arias, M., 2016b. Role of dopamine neurotransmission in the long-term effects of repeated social defeat on the conditioned rewarding effects of cocaine. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 311, 864–868. https://doi.org/10.1016/j.pnpbp.2016.07.008.

Nasanbuyan, N., Yoshida, M., Takayanagi, Y., Inutsuka, A., Nishimori, K., Yamanaka, A., Onaka, T., 2018. Oxytocin-oxytocin receptor systems facilitate social defeat posture in male mice. Endocrinology 159, 763–775. https://doi.org/10.1210/en.2017-00606.

Nestler, E.J., Carlezon, W.A., 2006. The mesolimbic dopamine reward circuit in depression. Biol. Psychiatry 59, 1151–1159. https://doi.org/10.1016/j.biopsych.2005.09.018.

Neumann, I.D., Slattery, D.A., 2016. Oxytocin in general anxiety and social fear: a translational approach. Biol. Psychiatry 79, 213–221. https://doi.org/10.1016/j.biopsych.2015.06.004.

Neumann, I.D., Wigger, A., Torner, L., Holsboer, F., Landgraf, R., 2000. Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. J. Neuroendocrinol. 12, 235–243. https://doi.org/10.1046/j.1365-2826.2000.00442.x.

Nikulina, E.M., Johnston, C.E., Wang, J., Hammer, R.P., 2014. Neurotrophins in the ventral tegmental area: role in social stress, mood disorders and drug abuse. Neuroscience 282, 122–138. https://doi.org/10.1016/j.neuroscience.2014.05.028.

Nikulina, E.M., Lacagnina, M.J., Fanous, S., Wang, J., Hammer, R.P., 2012. Intermittent social defeat stress enhances mesocorticolimbic ΔFosB/BDNF co-expression and persistently activates corticotegmental neurons: implication for vulnerability to psychostimulants. Neuroscience 212, 38–48. https://doi.org/10.1016/j.neuroscience.2012.04.012.

Numan, S., Lane-Ladd, S.B., Zhang, L., Lundgren, K.H., Russell, D.S., Seroogy, K.B., Nestler, E.J., 1998. Differential regulation of neurotrophin and trk receptor mRNAs in catecholaminergic nuclei during chronic opiate treatment and withdrawal. J. Neurosci. 18, 10700–10708. https://doi.org/10.1523/jneurosci.18-24-10700.1998.

Onaka, T., Takayanagi, Y., Yoshida, M., 2012. Roles of oxytocin neurones in the control of stress, energy metabolism, and social behaviour. J. Neuroendocrinol. 24, 587–598. https://doi.org/10.1111/j.1365-2826.2012.02300.x.

Parker, K.J., Buckmaster, C.L., Schatzberg, A.F., Lyons, D.M., 2005. Intranasal oxytocin administration attenuates the ACTH stress response in monkeys. Psychoneuroendocrinology 30, 924–929. https://doi.org/10.1016/j.psyneuen.2005.04.002.

Pedersen, C.A., Smedley, K.L., Leserman, J., Jarskog, L.F., Rau, S.W., Kampov-Polevoi, A., Casey, R.L., Fender, T., Garbutt, J.C., 2013. Intranasal oxytocin blocks alcohol withdrawal in human subjects. Alcohol Clin. Exp. Res. 37, 484–489. https://doi.org/10.1111/j.1530-0277.2012.01958.x.

Peters, S., Slattery, D.A., Uschold-Schmidt, N., Reber, S.O., Neumann, I.D., 2014. Dose-dependent effects of chronic central infusion of oxytocin on anxiety, oxytocin receptor binding and stress-related parameters in mice. Psychoneuroendocrinology 42, 225–236. https://doi.org/10.1016/j.psyneuen.2014.01.021.

Petrovic, P., Kalisch, R., Singer, T., Dolan, R.J., 2008. Oxytocin attenuates affective evaluations of conditioned faces and amygdala activity. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.4572-07.2008.

Pizarro, J.M., Lumley, L.A., Medina, W., Robison, C.L., Chang, W.E., Alagappan, A., Bah, M.J., Dawood, M.Y., Shah, J.D., Mark, B., Kendall, N., Smith, M.A., Saviolakis, G.A., Meyerhoff, J.L., 2004. Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. Brain Res. 1025, 10–20. https://doi.org/10.1016/j.brainres.2004.06.085.

Purba, J.S., Hoogendijk, W.J.G., Hofman, M.A., Swaab, D.F., 1996. Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. Arch. Gen. Psychiatr. 53, 137–143. https://doi.org/10.1001/archpsyc.1996.01830020055007.

Qi, J., Yang, J.Y., Wang, F., Zhao, Y.N., Song, M., Wu, C.F., 2009. Effects of oxytocin on methamphetamine-induced conditioned place preference and the possible role of glutamatergic neurotransmission in the medial prefrontal cortex of mice in reinstatement. Neuropharmacology 56, 856–865. https://doi.org/10.1016/j.neuropharm.2009.01.010.

Quadros, I.M.H., Miczek, K.A., 2009. Two modes of intense cocaine bingeing: increased persistence after social defeat stress and increased rate of intake due to extended access conditions in rats. Psychopharmacology 206, 109–120. https://doi.org/10.1007/s00213-009-1584-6.

Reguilón, M.D., Montagud-Romero, S., Ferrer-Pérez, C., Roger-Sánchez, C., Aguilar, M.A., Miñarro, J., Rodríguez-Arias, M., 2017. Dopamine D2receptors mediate the increase in reinstatement of the conditioned rewarding effects of cocaine induced by acute social defeat. Eur. J. Pharmacol. 799, 48–57. https://doi.org/10.1016/j.ejphar.2017.01.039.

Ring, R.H., Malberg, J.E., Potestio, L., Ping, J., Boikess, S., Luo, B., Schechter, L.E., Rizzo, S., Rahman, Z., Rosenzweig-Lipson, S., 2006. Anxiolytic-like activity of oxytocin in male mice: behavioral and autonomic evidence, therapeutic implications. Psychopharmacology 185, 218–225. https://doi.org/10.1007/s00213-005-0293-z.

Rodríguez-Arias, M., Castillo, A., Daza-Losada, M., Aguilar, M.A., Miñarro, J., 2009. Effects of extended cocaine conditioning in the reinstatement of place preference. Physiol. Behav. 96, 620–630. https://doi.org/10.1016/j.physbeh.2008.12.011.

Rodríguez-Arias, M., Miñarro, J., Aguilar, M.A., Pinazo, J., Simón, V.M., 1998. Effects of risperidone and SCH 23390 on isolation-induced aggression in male mice. Eur. Neuropsychopharmacol 8, 95–103. https://doi.org/10.1016/S0924-977X(97)00051-5.

Rodríguez-Arias, M., Montagud-Romero, S., Rubio-Araiz, A., Aguilar, M.A., Martín-García, E., Cabrera, R., Maldonado, R., Porcu, F., Colado, M.I., Miñarro, J., 2017. Effects of repeated social defeat on adolescent mice on cocaine-induced CPP and self-administration in adulthood: integrity of the blood–brain barrier. Addict. Biol. 22, 129–141. https://doi.org/10.1111/adb.12301.

Rodríguez-Arias, M., Navarrete, F., Blanco-Gandía, M.C., Arenas, M.C., Bartoll-Andrés, A., Aguilar, M.A., Rubio, G., Miñarro, J., Manzanares, J., 2016. Social defeat in adolescent mice increases vulnerability to alcohol consumption. Addict. Biol. 21, 87–97. https://doi.org/10.1111/adb.12184.

Sarnyai, Z., Babarczy, E., Kriván, M., Szabó, G., Kovács, G.L., Barth, T., Telegdy, G., 1991. Selective attenuation of cocaine-induced stereotyped behaviour by oxytocin: putative role of basal forebrain target sites. Neuropeptides 19, 51–56. https://doi.org/10.1016/0143-4179(91)90073-R.

Sarnyai, Z., Kovács, G.L., 1994. Role of oxytocin in the neuroadaptation to drugs of abuse. Psychoneuroendocrinology 19, 85–117. https://doi.org/10.1016/0306-4530(94)90062-0.

Sarnyai, Z., Kovács, G.L., 2014. Oxytocin in learning and addiction: from early discoveries to the present. Pharmacol. Biochem. Behav. 119, 3–9. https://doi.org/10.1016/j.pbb.2013.11.019.

Sarnyai, Z., Szabó, G., Kovacs, G.L., Telegdy, G., 1990. Oxytocin attenuates the cocaine-induced exploratory hyperactivity in mice. Neuroreport 1, 200–202. https://doi.org/10.1097/00001756-199011000-00006.

Sarnyai, Z., Szabó, G., Kovács, G.L., Telegdy, G., 1992. Opposite actions of oxytocin and vasopressin in the development of cocaine-induced behavioral sensitization in mice. Pharmacol. Biochem. Behav. 43, 491–494. https://doi.org/10.1016/0091-3057(92)90182-F.

Selten, J.P., Van Der Ven, E., Rutten, B.P.F., Cantor-Graae, E., 2013. The social defeat hypothesis of schizophrenia: an update. Schizophr. Bull. 39, 1180–1186. https://doi.org/10.1093/schbul/sbt134.

Sinha, R., Shaham, Y., Heilig, M., 2011. Translational and reverse translational research on the role of stress in drug craving and relapse. Psychopharmacology 218, 69–82. https://doi.org/10.1007/s00213-011-2263-y.

Slattery, D.A., Neumann, I.D., 2010. Chronic icv oxytocin attenuates the pathological high anxiety state of selectively bred Wistar rats. Neuropharmacology 58, 56–61. https://doi.org/10.1016/j.neuropharm.2009.06.038.

Smith, A.S., Wang, Z., 2014. Hypothalamic oxytocin mediates social buffering of the stress response. Biol. Psychiatry 1–8. https://doi.org/10.1016/j.biopsych.2013.09.017.

Soria, G., Barbano, M.F., Maldonado, R., Valverde, O., 2008. A reliable method to study cue-, priming-, and stress-induced reinstatement of cocaine self-administration in mice. Psychopharmacology 199, 593–603. https://doi.org/10.1007/s00213-008-1184-x.

Steketee, J.D., Kalivas, P.W., 2011. Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. Pharmacol. Rev. 63, 348–365. https://doi.org/10.1124/pr.109.001933.

Taylor, S.L., Stanek, L.M., Ressler, K.J., Huhman, K.L., 2011. Differential brain-derived neurotrophic factor expression in limbic brain regions following social defeat of territorial aggression. Behav. Neurosci. 125, 911–920. https://doi.org/10.1037/a0026172.

Thomas, M.J., Kalivas, P.W., Shaham, Y., 2008. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. Br. J. Pharmacol. https://doi.org/10.1038/bjp.2008.77.

Tornatzky, W., Miczek, K.A., 1993. Long-term impairment of autonomic circadian rhythms after brief intermittent social stress. Physiol. Behav. 53, 983–993. https://doi.org/10.1016/0031-9384(93)90278-N.

Tsai, S.J., 2007. Increased central brain-derived neurotrophic factor activity could be a risk factor for substance abuse: implications for treatment. Med. Hypotheses 68, 410–414. https://doi.org/10.1016/j.mehy.2006.05.035.

Tsankova, N.M., Berton, O., Renthal, W., Kumar, A., Neve, R.L., Nestler, E.J., 2006. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat. Neurosci. 9, 519–525. https://doi.org/10.1038/nn1659.

Tzschentke, T.M., 2007. Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict. Biol. 12, 227–462. https://doi.org/10.1111/j.1369-1600.2007.00070.x.

Unternaehrer, E., Luers, P., Mill, J., Dempster, E., Meyer, A.H., Staehli, S., Lieb, R., Hellhammer, D.H., Meinlschmidt, G., 2012. Dynamic changes in DNA methylation of stress-associated genes (OXTR, BDNF) after acute psychosocial stress. Transl. Psychiatry 2. https://doi.org/10.1038/tp.2012.77.

Vidal-Infer, A., Arenas, M.C., Daza-Losada, M., Aguilar, M.A., Miñarro, J., Rodríguez-Arias, M., 2012. High novelty-seeking predicts greater sensitivity to the conditioned rewarding effects of cocaine. Pharmacol. Biochem. Behav. 102, 124–132. https://doi.org/10.1016/j.pbb.2012.03.031.

Waldherr, M., Neumann, I.D., 2007. Centrally released oxytocin mediates mating-induced anxiolysis in male rats. Proc. Natl. Acad. Sci. Unit. States Am. 04, 16681–16684. https://doi.org/10.1073/pnas.0705860104.

Wang, Q., Shao, F., Wang, W., 2018. Region-dependent alterations in cognitive function and ERK1/2 signaling in the PFC in rats after social defeat stress. Neural Plast. 2018. https://doi.org/10.1155/2018/9870985.

Windle, R.J., Shanks, N., Lightman, S.L., Ingram, C.D., 1997. Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138, 2829–2834. https://doi.org/10.1210/endo.138.7.5255.

Wotjak, C.T., Kubota, M., Liebsch, G., Montkowski, A., Holsboer, F., Neumann, I., Landgraf, R., 1996. Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J. Neurosci. 16, 7725–7732. https://doi.org/10.1110/ps.8.3.529.

Xu, H., Wang, J., Zhang, K., Zhao, M., Ellenbroek, B., Shao, F., Wang, W., 2018. Effects of adolescent social stress and antidepressant treatment on cognitive inflexibility and Bdnf epigenetic modifications in the mPFC of adult mice. Psychoneuroendocrinology 88, 92–101. https://doi.org/10.1016/j.psyneuen.2017.11.013.

Yang, B., Ren, Q., Ma, M., Chen, Q.X., Hashimoto, K., 2016a. Antidepressant effects of (+)-MK-801 and (-)-MK-801 in the social defeat stress model. Int. J. Neuropsychopharmacol. 19, 1–5. https://doi.org/10.1093/ijnp/pyw080.

Yang B, Zhang JC, Han M, Yao W, Yang C, Ren Q, Ma M, Chen QX, Hashimoto K. 2016b. Comparison of R-ketamine and rapastinel antidepressant effects in the social defeat stress model of depression. Psychopharmacology. 233, 3647–3657. https://doi.org/10.1007/s00213-016-4399-2.

Yap, J.J., Chartoff, E.H., Holly, E.N., Potter, D.N., Carlezon, W.A., Miczek, K.A., 2015. Social defeat stress-induced sensitization and escalated cocaine self-administration: the role of ERK signaling in the rat ventral tegmental area. Psychopharmacology 232, 1555–1569. https://doi.org/10.1007/s00213-014-3796-7.

Yoshida, M., Takayanagi, Y., Inoue, K., Kimura, T., Young, L.J., Onaka, T., Nishimori, K., 2009. Evidence that oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice. J. Neurosci. 29, 2259–2271. https://doi.org/10.1523/JNEUROSCI.5593-08.2009.

Zanos, P., Wright, S.R., Georgiou, P., Yoo, J.H., Ledent, C., Hourani, S.M., Kitchen, I., Winsky-Sommerer, R., Bailey, A., 2014. Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine A2A receptor-independent mechanism. Pharmacol. Biochem. Behav. 119, 72–79. https://doi.org/10.1016/j.pbb.2013.05.009.

Zhou, L., Sun, W.L., Young, A.B., Lee, K., McGinty, J.F., See, R.E., 2014. Oxytocin reduces cocaine seeking and reverses chronic cocaine-induced changes in glutamate receptor function. Int. J. Neuropsychopharmacol. 18. https://doi.org/10.1093/ijnp/pyu009.