NEUROBIOLOGICAL LINKS BETWEEN DEPRESSION AND DRUG DEPENDENCE

Irene Gracia Rubio

DOCTORAL THESIS UPF/ 2016

Thesis Supervisor:

Olga Valverde Granados MD PhD

DEPARTAMENT DE CIÈNCES EXPERIMENTALS I DE LA SALUT

Universitat Pompeu Fabra Barcelona

La Dra. Olga Valverde Granados, Catedrática de Psicobiología del Departament de Ciències Experimentals i de la Salut de la Universitat Pompeu Fabra

Hace constar

Que la tesis doctoral presentada por Irene Gracia Rubio, con el título "NEUROBIOLOGICAL LINKS BETWEEN DEPRESSION AND DRUG DEPENDENCE", ha sido realizada bajo su dirección. Tras haberla examinado hace constar su autorización para que se realicen los trámites que conduzcan a su presentación y defensa.

Y para que conste a los efectos oportunos, firma el presente documento en Barcelona a ___ de _____ del 2016.

Fdo. Dra. Olga Valverde Granados

This thesis doctoral was supported by MINECO (SAF2013-41761-R-FEDER), Ministry of Health (Retic-ISCIII-RD/12/0028/0024-FEDER and *Plan Nacional sobre Drogas* 2014/020), and *Generalitat de Catalunya* (2014SGR34). IG-R was funded by FPI fellowship BES-2011-046655 associated with SAF2010-15793.

A mis padres, a la memoria de mi abuela Irene, a Pablo

"*Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa"*

Mahatma Gandhi

AGRADECIMIENTOS

Cuantas veces he pensado en el momento en el que escribiría los agradecimientos, y por fin aquí estoy, escribiendo el último apartado de la tesis. Hace 4 años y pico que comenzó esta historia que conllevó al cambio de ciudad, cambio de trabajo…vamos un cambio de vida en toda regla. Al principio me costó adaptarme pero poco a poco conseguí hacerme a la vida del nuevo grupo y a la de Barcelona. En estos 4 años ha habido momentos buenísimos y momentos muy malos, a los que nunca pensé que debería hacer frente y afortunadamente siempre he tenido a mi lado gente con quien celebrar y en la que apoyarme para superar los baches. Espero no dejarme a nadie a quién agradecer.

Primero quiero darle las gracias a Olga, mi directora de tesis. Gracias por darme la oportunidad de realizar la tesis en GReNeC. Esta tesis me ha dado la oportunidad de crecer como persona, y darme cuenta de que con esfuerzo y trabajo todo (bueno, casi todo) se puede conseguir. ¡Muchas gracias!

A las segundas personas a las que quiero agradecer son a mis compañeras y amigas, Maria, Neus y Anna, de lo mejorcito que me llevo de esta etapa. Maria, juntas empezamos y casi juntas acabamos, qué te voy a decir que no sepas ya, si esta tesis es casi tuya también. Mil gracias por el par de manos extra (y cerebro, xD), siempre estas dispuesta a ayudarme, aconsejarme, criticarme (en el buen sentido), SIEMPRE. Hacemos muy buen equipo, ¿no? Recuerda que eres muy grande. Neus, gracias por tu ayuda y por todos los consejos que me has dado. La experiencia es el mejor consejo pero siempre llega tarde, tú has sido la voz de la experiencia que me ha ayudado durante todos estos años y que me ha buscado soluciones a mis problemas cuando tú no podías ayudarme. ¡Gracias!. Anna, cuantas horas frustradas hemos pasado en el estabulario…pero mira al final lo conseguimos. Muchas gracias por no dejar que nos rindiéramos, por aconsejarme cuando lo he necesitado, por todo. Nenas, muchas gracias por apoyarme siempre dentro y fuera del labo y sobre todo por vuestra amistad que espero que la conservemos por muchas vueltas que demos, yo haré todo lo posible. También agradecer al grupo del "Barranquillo" por todas las cervezas que hemos tomado. Lucas, Pol, Jordi ¡gracias!

También al resto de personas que están en el grupo, Marta, Miguel Ángel, Vincent, Anna, Adriana y especialmente a Lidia. Lidia, gracias por traer tantas ganas y motivación, ha sido un placer trabajar contigo y estate tranquila que al final las cosas siempre acaban saliendo. Mucho ánimo en lo que te queda de camino y ya sabes que puedes contar conmigo. Y también a la gente que pasó por el grupo. Gracias Emily por todas las discusiones científicas que hemos tenido y tu rigor científico. Gracias a Clara también.

Gracias a Roser Nadal por ayudarme con los experimentos de "Maternal care" y por tus comentarios. Me han ayudado a plantearme puntos de vista diferentes.

También quería agradecer a Óscar Pozo y Josep Marcos, por su colaboración en mi proyecto y por la manera que tienen de plantearse la ciencia. Muchas gracias por analizar mis muestras, ha sido un placer trabajar con vosotros.

Muchas gracias al grupo de María Luisa Laorden y María Victoria Milanés por vuestra colaboración en mi proyecto. Gracias por saber exprimir al máximo mis muestras y obtener tan buenos resultados.

I also want to thank Emmanuel Valjent for giving me the opportunity of working in his lab and also to the rest of the team, Emma, Anne, Jihane and Eve. I really enjoyed my 3-month stay in Montpellier and I learnt a lot. I love the way you do research and solve your problems! Especialmente a Emma y a Miquel por tratarme como si me conocierais de toda la vida desde el primer día, sin vosotros no hubiera sido igual. Emma, mil gracias por todo lo que me has enseñado, eres la motivación y perfección en persona, ¡este año a por todas!

Gracias a Elena Escubedo y todo su grupo por dejarnos formar parte de su proyecto. Especialmente a Raúl, gracias por tu ayuda y apoyo.

Gracias también al grupo de Neurophar por estar siempre dispuestos a echarnos una mano. Especialmente a Dulce y Cris. África, mil gracias por tu clase magistral (nos cambió la vida) y por estar siempre dispuesta a resolver nuestras dudas, que no fueron pocas. También en esta etapa final de la tesis a Laura, siempre disponible para contestar a mis preguntas.

Ahora toca el turno de agradecer a mis amigos de toda la vida, César, Laura, Palma, Dulce, Elena y Yessi. Gracias por escucharme, por animarme…y sobre todo por esta siempre cuando lo necesito. De verdad, ¡mil gracias! También a las incorporaciones Gaizka, Borja y Guillermo, gracias por cuidar tan bien a mis chicas y formar parte de esta pequeña familia. Por último y por eso no menos importante, a Carla que nos alegras los días con tus sonrisas.

A mis amigos de Montalbán, Bego, Blanca, Elena, Dani, Primi, Raquel, Tania y Villi. Muchas gracias por todo vuestro apoyo que aunque nos veamos poquito me acuerdo muchísimo de vosotros. También a la compañía Javi, Rafa, Álvaro, Alex y Pablo. Finalmente, gracias a Daniel, la última incorporación a la peña Bacarrá.

A mis amigos de Zaragoza. Primero mis chicas, Tere, Intza, Eva, Lau, Maru y Lore. Muchas gracias por ser mi familia durante 7 años. Me encanta que aunque pase el tiempo y nos veamos muy poco nuestra relación no cambie. Y muchas gracias también por estar siempre que lo necesito. A los chicos, Hacker, Valerón, Ángel, Javi, Adrián, Perchas, Machi y Pablo muchas gracias por vuestro apoyo siempre y en especial durante este periodo. Mil gracias por las quedadas que hacemos, me hacen volver a los años de universidad y olvidarme de todo.

A mis amigos de la uni, Javi, María, Sonia y especialmente a Bea por entenderme tan bien, apoyarme y ayudarme en todo, mil gracias. También a Sara, que aunque desde que terminamos la carrera no nos hemos visto, siempre está allí.

Al grupo de la bodegueta, Miqui, Patri, Roldán, Camino e Izás. Gracias por sacarme de casa en esta última época y también a la bodegueta por ser tan asequible. Camino e Izás, gracias por todo, ¡sois los mejores vecinos del mundo!

A los amigos de Alcañiz, muchas gracias por tratarme como una más desde el primer momento, por escucharme y animarme. Gracias por vuestro apoyo.

Muchas gracias a Teresa y Jesús A por apoyarnos en todas nuestras decisiones, creer en nosotros y por todo vuestro apoyo en esta recta final.

También a Jesús F y Yurena y al resto de familia porque ya formáis parte de la mía.

A mis primos Ragüel y Fernando y también a Sara. Porque ya sabéis lo importante que sois para mí, quería daros las gracias por todo vuestro apoyo. También agradecer al resto de mi familia.

A mi abuela Irene, que justo se fue cuando esta historia comenzaba. Sé que me hubieras apoyado en todo, como siempre lo hacías. No pasa un día en que no me acuerde de ti. Gracias por todo, yaya.

A Zoila y José María, mis padres. Tengo tanto que agradeceros que no se ni por dónde comenzar. Gracias por ser como sois y por hacerme la persona que soy hoy en día, aunque sé que no os lo he puesto fácil. Por apoyarme incondicionalmente, por aconsejarme, por animarme, por creer en mí y por no dejar que me rinda jamás. Mamá, papá, muchas gracias por todo, os quiero muchísimo.

Y finalmente a Pablo, mi amigo, mi pareja. A ti, que has sufrido esta tesis tanto como yo, que te has quedado los fines de semana conmigo, que me has apoyado, animado y sobre todo me has ayudado a llegar al final. Por quedarte a mi lado siempre. Por las etapas que llevamos y las que nos quedan por vivir, muchas gracias por todo cariño. ¡Te quiero mil!

¡MUCHÍSIMAS GRACIAS A TODOS!

ABSTRACT

Early life experiences play a key role in brain function and behaviour. Adverse experiences during childhood are consequently a risk factor for the ulterior development of psychiatric diseases that may persist into adulhood. Maternal separation is a validated mouse model for maternal neglect, producing negative early life experiences that result in subsequent emotional alterations. In addition, social environmental enrichment has been proposed as a protective factor that may reduce the vulnerability of individuals to suffer psychiatric disorders. Therefore, the communal nest model confers resilience against despair-like behaviour in mice. Moreover, early-life stress enhances the vulnerability to develop substance use disorders, principally during adolescence since the brain is under a maturation process. Hence, depressive states are associated with drug use disorders and abuse vulnerability since depressive patients could consume drugs to alleviate their symptoms.

The **general objective** of this thesis was to investigate the short and long-term consequences of the exposure to a procedure of early life neglect and social enrichment on emotional alterations and drug abuse. Therefore, behavioural studies and neurochemical parameters were assessed to elucidate our main hypothesis.

Our results demonstrated that maternal separation is an appropriate model for studying the implication of depression in the vulnerability of substance use since this model induces mood alterations in adolescence that persist into adulthood as well as neurochemical alterations including neuroinflammation and the unbalanced of tryptophan-kynurenine metabolism pathways. However, under our experimental conditions, we cannot confirm the protective role of communal nest due to the behavioural alterations found in these mice, probably because this

breeding condition involves energetic cost and increases the competition for food in pups.

Regarding the cocaine effects, mice exposed to the maternal separation showed attenuated rewarding properties of cocaine and decreased behavioural sensitization, whereas no changes were found in self-administration of cocaine. The evaluation of neuroplasticity in the striatal dopaminergic pathways revealed decreased protein expression levels of D2 receptors and increased of the transcriptional factor Nurr1, suggesting a hypofunctionality of the dopaminergic system accordingly with the anhedonia-like behaviour observed in mice exposed to maternal separation.

In **conclusion**, our results lead us to propose that detrimental early life events such as maternal neglect reproduces most of the behavioural and neurochemical alterations associated with emotional disorders in mice. In addition, maternal separation could be considered to be useful to study the comorbidity between depression and substance use disorder since induces alterations in emotional and drug addictive behaviours.

RESUMEN

Las experiencias tempranas tienen un papel importante en el desarrollo cerebral, pudiendo afectar al control de la respuesta emocional. En particular, los eventos adversos durante la infancia representan factores de riesgo para el posterior desarrollo de enfermedades psiquiátricas en individuos vulnerables. En este sentido, la separación maternal es un modelo validado en roedores para simular la situación de abandono materno, produciendo una situación de estrés crónico en la edad temprana que conlleva alteraciones emocionales a largo plazo. Asimismo, el modelo de enriquecimiento de ambiente social se ha propuesto como una situación protectora que podría reducir la vulnerabilidad para sufrir desórdenes psiquiátricos. De hecho, el modelo de nido comunal confiere resistencia a la conducta de desesperanza en el ratón. Además, el estrés crónico en las épocas tempranas de la vida aumenta la vulnerabilidad a desarrollar trastornos por uso de sustancias, principalmente durante la adolescencia, cuando el cerebro se encuentra en un proceso de maduración. Por consiguiente, los estados depresivos están asociados con una mayor vulnerabilidad para el uso de drogas puesto que los pacientes depresivos podrían consumir drogas para aliviar sus síntomas.

El **objetivo general** de esta tesis ha sido estudiar las consecuencias a corto y largo plazo de la exposición temprana a un estrés crónico versus un enriquecimiento de ambiente social sobre la respuesta emocional y el consumo de cocaína. Para abordar nuestra hipótesis principal hemos desarrollado estudios comportamentales y neuroquímicos.

Nuestros resultados demuestran que la separación maternal es un modelo apropiado para el estudio de la participación de la depresión en la vulnerabilidad al uso de sustancias, ya que este modelo produce alteraciones emocionales durante la adolescencia que se mantienen en la

etapa adulta, así como cambios neuroquímicos que incluyen neuroinflamación y un desequilibrio en la ruta metabólica del triptófano y la kinurenina. Sin embargo, no podemos confirmar el papel protector del nido en comuna debido a las alteraciones comportamentales encontradas en nuestras condiciones experimentales, probablemente a causa de que este modelo de cría conlleva costes energéticos y aumenta la competición por comida entre las crías.

Respecto a los efectos de la cocaína, los ratones expuestos a la separación maternal mostraron una respuesta atenuada a los efectos reforzantes de la cocaína en el modelo de preferencia de lugar, así como una disminución en la sensibilización conductual, mientras que no se observaron cambios en la autoadministración de cocaína. Además, el estudio de la neuroplasticidad de la vía dopaminérgica en el estriado mostró una disminución en la expresión proteica del receptor D2, así como un aumento en la expresión del factor dopaminérgico Nurr1, sugiriendo una hipo-funcionalidad del sistema dopaminérgico de acuerdo con el comportamiento anhedónico observado en los ratones expuestos a la separación maternal.

En **conclusión**, nuestros resultados demuestran que las experiencias adversas durante la etapa temprana de la vida, como la separación maternal, reproducen la mayoría de las alteraciones comportamentales y neuroquímicas relacionadas con depresión en el ratón. Asimismo, la separación maternal puede considerarse como un modelo conveniente para el estudio de la comorbilidad psiquiátrica entre depresión y el trastorno por uso de drogas, ya que reproduce alteraciones a nivel emocional y en el comportamiento motivacional de los animales.

INDEX

AGRADECIMIENTOS XIII

ABSTRACT XVII

RESUMEN XIX

INDEX XXI

ABBREVIATIONS XXVII

INTRODUCTION **1**

Emotional disorders: Depression 3

1. Epidemiology 3

2. Pathophysiology of depression 4

2.1. Monoaminergic hypothesis of depression 5

2.1.1. Serotonin 6

2.1.2. Noradrenaline 7

2.2. Other neurotransmitters alterations 8

2.3. Hypothalamic-Pituitary-Adrenal axis and depression 9

2.4. Neuronal plasticity in the pathophysiology of depression 12

2.5. Reward circuit and dopaminergic system during depression 14

2.6. Inflammatory reactions and oxidative stress in the pathophysiology of depression 15

3. Vulnerability factors influencing the development of depression 19

3.1. Genetic factors involved in depression 20

3.2. Epigenetic factors participating in depression 22

3.3. Sex and gender factors associated to depression 23

3.4. Environmental factors influencing depression 26

Early life experiences on the control of mood 28

1. Brain development 28
1.1. Brain development in the prenatal period 28
1.2. Brain development in the postnatal period 30
1.3. Brain development in the adolescence 33
2. Protective and detrimental factors influencing emotional reactions during childhood 35
2.1. Protective factors during childhood 35
2.1.1. Positive early experiences: Human evidences 35
2.1.2. Positive early experiences: Experimental studies 36
2.2. Detrimental factors during childhood 38
2.2.1. Adverse early life experiences: Human evidences 38
2.2.2. Adverse early life experiences: Experimental animal studies 41
3. Implications of early-life detrimental experiences on the development of depression 43
Drug of abuse and substances use disorders 47
1. Dopaminergic system in drug abuse 50
1.1. Metabolism of dopamine 53
1.2. Receptors of dopamine 54
1.3. Drug-induced adaptive changes in the dopaminergic system 54
2. Cocaine as a drug of abuse: history of it recreational use, epidemiology, and mechanism of action 56
2.1. Brief history of recreational use of cocaine 56
2.2. Epidemiology 57
2.3. Mechanism of action 58
2.4. Pharmacological effects 59
3. Cocaine abuse and depressive disorders 63

OBJETIVES **67**

METHODOLOGY **71**
1. Animals 73

2. Rearing conditions 73
3. Maternal care 75
4. Behavioural procedures 75
4.1. Spontaneous Locomotor Activity 77
4.2. Elevated plus maze 77
4.3. Tail suspension test 78
4.4. Saccharin test 79
4.5. Electrical nociceptive threshold 80
4.6. Passive avoidance test 81
5. Studies conducted to evaluate inflammatory reactions in mice exposed to different rearing conditions 83
5.1. Evaluation of peripheral inflammatory response. Interleukine-6 determination 83
5.2. Evaluation of neuroinflammatory responses. Astrocytes and microglia 83
5.3. Image analysis to evaluate immunohistochemical immunofluorescence studies 84
6. Tryptophan-kynurenine pathway analysis 85
6.1. Chemicals and reagents 85
6.2. Quantification of metabolites by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) 85
7. Studies to evaluate rewarding and addictive effects induced by cocaine 87
7.1. Drug and injection procedure 87
7.2. Conditioned Place Preference Paradigm 88
7.2.1. Procedure 88
7.2.2. Preparation of tissue extract 90
7.2.3. Dopaminergic system evaluation: Electrophoresis and Western Blotting 90
7.2.4. Estimation of DA and its metabolite DOPAC 91
7.3 Cocaine-induced locomotor sensitization procedure 92
7.4 Operant cocaine self-administration 94
7.4.1. Surgery for the i.v. catheter implantation 94
7.4.2. Apparatus for self-administration experiments 95

7.4.3. Drug self-administration procedure 95
8. Statistical analysis 96

RESULTS **99**
1. Spontaneous maternal care evaluation 101
2. Effects of rearing conditions on body weight 105
3. Effects of rearing conditions on the emotional behaviour 106
3.1. Effects of rearing conditions on locomotor activity 106
3.2. Effect of rearing conditions on anxiety-like behaviour in the elevated plus maze 108
3.3. Effect of rearing conditions on despair behaviour in the tail suspension test 110
3.4. Effect of rearing conditions on anhedonia evaluated with the saccharin test 112
3.5. Nociceptive threshold 115
3.6. Effect of rearing conditions on emotional memory evaluated in the passive avoidance paradigm 116
4. Effect of rearing conditions on inflammatory reactions 118
4.1. Peripheral inflammatory response. Serum level of the cytokine IL-6 118
4.2. Neuroinflammatory reactions in discrete brain areas 118
4.2.1. Microglia activation. Iba1 immunostaining 118
4.2.2. Astrocytes. GFAP inmunostaining 121
5. Rearing conditions produces alterations in the tryptophan-kynurenine metabolic pathway 122
6. Influence of maternal separation on rewarding and addictive properties of cocaine 127
6.1. Evaluation of rewarding properties of cocaine in the conditioned place preference paradigm 127
6.2. Expression levels of DAT and D2R proteins and DA turnover in the NAc 128
6.3. Expression levels of Nurr1 and Pitx3 proteins in the VTA 131
6.4. Cocaine-induced locomotor sensitization 132
6.5. Reinforcing effects of cocaine in the self-administration paradigm 136

DISCUSSION **139**

1. The evaluation of early experiences using animal models 142

2. Early life experiences on emotional behaviour, inflammatory response and tryptophan-kynurenine pathway. 143

3. Maternal separation influences cocaine-addictive behaviour through the modulation of the dopaminergic system. 158

CONCLUSIONS **167**

REFERENCES **171**

ANNEX **223**

Article 1 **225**

Maternal separation induces neuroinflammation and long-lasting emotional aterations in mice

Gracia-Rubio I, Moscoso-Castro M , Pozo OJ, Marcos J, Nadal R, Valverde O

Prog Neuropsychopharmacol Biol Psychiatry. 2015 65:104-17

Article 2 **241**

Maternal separation alters cocaine addiction behaviour through dopaminergic system.

Gracia-Rubio I, Martinez-Laorden E, Moscoso-Castro M, Milanés MV, Laorden MV, Valverde O

Unsent

Article 3 **283**

Ethanol binge drinking enhances cocaine-induced sensitization and reinforcement in adolescent mice

Esteve-Arenys A, Gracia-Rubio I, Cantacorps L, Pozo O J, Marcos J, Rodríguez-Árias M, Miñarro J, Valverde O

Submitted Addict Biolg.

Article 4 **287**

Genetic blockade of adenosine A2A receptors induces cognitive impairments and anatomical changes related to psychotic symptoms in mice.

Moscoso-Castro M, Gracia-Rubio I, Ciruela F, Valverde O

Submitted Eur Neuropsychopharmacol

Article 5 **291**

Targeting Tryptophan and Tyrosine Metabolism by Liquid Chromatography Tandem Mass Spectrometry.

Marcos J, Renau N, Valverde O, Aznar-Laín G, Gracia-Rubio I, Gonzalez-Sepulveda M , Pérez-Jurado LA, Ventura R, Segura J, Pozo OJ

Journal of Chromatography A Doi: 10.1016/j.chroma.2016.01.023

Article 6 **295**

Modulation of cAMP-specific PDE without emetogenic activity: new sulfide-like PDE7 inhibitors.

García AM, Brea J, Morales-García JA, Perez DI, González A, Alonso-Gil S, Gracia-Rubio I, Ros-Simó C, Conde S, Cadavid MI, Loza MI, Perez-Castillo A, Valverde O, Martinez A, Gil C.

J Med Chem. 2014 57(20):8590-607

ABBREVIATIONS

3-MT	3-methoxytryamine
5-HT	Serotonin
5-HTA1	Serotonin 1A receptor
5HTTP	Serotonin transporter
AADC	Aromatic L-amino acid decarboxylase
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipotrein E
ATP	Adenosine-5-triphosphate
BDNF	Brain-derived neurotrophic factor
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
cAMP	Cyclic adenosine monophosphate
CN	Communal nest
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CPP	Conditioned place preference
CRF	Corticotropin-releasing factor
D1R	D1 dopamine receptor
D2R	D2 dopamine receptor
DA	Dopamine
DAT	Dopamine transporter
DG	Dentate gyrus
DMS-5	Diagnostic and Statistical Manual of Mental Disorders fifth edition
DOPAC	Dihydroxyphenylacetic acid
DβH	Dopamine β-hydroxilase
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated pluz maze
GABA	Gamma-aminobutyric acid
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acid protein
GNB3	Guanine nucleotide binding protein (G protein), beta polypeptide 3
GW	Gestation week

GWAS	Genome wide association studies
HC	Hippocampus
High-LG	High-licking and grooming
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HVA	Homovanillic acid
i.p.	Intraperitoneal
i.v.	Intravenous
Iba1	Ionized calcium-binding adapter molecule 1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
KO	Knockout
KYN	Kynurenine
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Low-LG	Low-licking and grooming
LTD	Long-term depression
LTP	Long-term potentiation
MAO	Monoamine oxidase
MSEW	Maternal separation with early weaning
MTHFR	Methylenetetrahydrofolate reductase
NAc	Nucleus accumbens
NA	Noradrenaline
NF-kB	Nuclear factor-kB
NMDA	N-metil-D-aspartato
Nr3C1	Nuclear Receptor Subfamily 3, Group C, Member 1 (Glucocorticoid Receptor)
Nurr1	Nuclear receptor-related factor 1
PB	Phosphate buffer
PBS	Phosphate buffer saline
PD	Postnatal day
PFC	Prefrontal cortex
Pitx3	Paired-like homeodomain 3
PNMT	Phenylethanolamine N-methyltransferase
PTSD	Posttraumatic stress disorder
Rac1	Ras-related C3 botulinum toxin substrate 1

ROS	Reactive oxygen species
s.c.	Subcutaneous
SDS	Sodium dodecyl sulfate
SN	Standard nesting
SRM	Selected reaction monitoring
SYN1	Synapsin 1
TH	Tyrosine hydroxylase
THC	Tetrahidrocannabinol
TNF-α	Tumour necrosis factor-α
TRP	Tryptophan
TST	Tail suspension test
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WHO	World Health Organization

INTRODUCTION

Emotional disorders: Depression

1. Epidemiology

Emotional disorders, including depression, are the most prevalent psychiatric disorders worldwide contributing to the global burden of disease (Murray and Lopez 2013). Nowadays, the World Health Organization (WHO) estimates in 350 million the people affected by depression and predicts that depressive disorders will be the greatest contributor to the global burden of disease by 2030 (WHO, 2015). Moreover, the WHO has ranked depression as the leading cause of disability worldwide (Mathers et al. 2005; Stuart and Baune 2014) and it is the most costly brain disorder in Europe (Gabilondo et al. 2010). Regarding socio-demographic data, epidemiological studies found that the risk of suffering depression is double in woman than in men (Weissman et al. 1996; Kudielka and Kirschbaum 2005; Sherin and Nemeroff 2011).

In Spain, depression affects 4-5% of the population, rising to 10% when considered also the no diagnosed patients. The risk of suffering an episode along lifespan is two times higher in women than men (16.5% vs. 8.9%). Furthermore, the prevalence of depression is increasing in Spain and in Europe. This raise in the prevalence may be due to the increasing medium age of the population, the stress level in our society as well as the consumption of substance of abuse (Mental Health Strategy of the National Health System, 2009-2013 Ministry of Health).

In addition, only 50% of people suffering depression receive treatment and more than a quarter of depressive treated patients fail to achieve remission despite trying multiple treatments (Al-Harbi 2012; Felger and Lotrich 2013). The treatment-resistance induces a decline in physical health in these patients increasing the health care utilization (Al-Harbi

2012). Moreover, an important percentage of patients relapse (Raedler 2011; Al-Harbi 2012), highlighting the need for more effective therapies.

2. Pathophysiology of depression

Depression, as stated above, is one of the most predominant psychiatric diseases (Murray and Lopez 2013), which manifests with symptoms at psychological, behavioural and physiological levels (Chopra et al. 2011; American Psychiatric Association 2013). This disorder is clinically characterized by deep deregulation of mood and affective behaviours and cognitive impairments, sleep and appetite alterations, fatigue, sexual dysfunction, and many other disturbances which include metabolic, endocrine or inflammatory alterations (Krishnan and Nestler 2008; Villanueva 2013). Therefore, the presentation of symptoms in depressive patients exhibits great variability and often involves opposite symptoms. Indeed, the Diagnostic and Statistical Manual of Mental Disorders fifth edition (DMS-5) includes symptoms for depression such as agitation or psychomotor retardation, insomnia or hypersomnia and significant weight loss or gain. DMS-5 also includes depressive mood, loss of interest or pleasure, loss of energy or fatigue, worthlessness or indecisiveness and thoughts of death, suicidal ideation or suicide attempt. The diagnostic criteria of depression involves meeting five (or more) of the above mentioned symptoms during 2 weeks, including depressive mood and diminished interest or pleasure (American Psychiatric Association 2013).

Consequently, depression is a multifactorial disorder and several hypotheses have been described trying to explain the mechanisms involved in the neurobiology of this disorder. However, the pathophysiology of depression is still unclear and it seems that a link between the different hypotheses may exist. Therefore, the main hypotheses associated with the pathophysiology of depression include the

monoaminergic theory which involves serotonin (5-HT), noradrenaline (NA) and dopamine (DA) neurotransmitters, although there are other neurotransmitters implicated such as glutamate and nitric oxide. Moreover, neuroplasticity mechanisms and reward system alterations are also involved in depression as well as the activation of the inflammatory response involving the immune system and oxidative stress mechanisms.

2.1. Monoaminergic hypothesis of depression

This theory was the earliest to appear, and is currently accepted (Coppen 1969; López-Muñoz and Alamo 2009; Artigas 2013). This hypothesis proposes that depression is caused by an imbalance in the monoaminergic neurotransmission including 5-HT, NA and DA systems (Palazidou 2012). The basis of this theory was focused in clinical and experimental data showing that reserpine, an antihypertensive drug, produced a depletion of presynaptic stores of 5-HT, NA and DA and induced depression-like behaviour (Prins et al. 2011). In contrast, euphoria and hyperactive behaviours were detected in patients being treated with ipronazid, a compound used for the treatment of tuberculosis, which increased brain concentrations of 5-HT and NA by inhibiting the monoamine oxidase enzyme (MAO) (López-Muñoz and Alamo 2009).

5-HT cell bodies are located mainly in the raphe nuclei, NA neurons are contained in the locus coeruleus and DA neurons are located in the ventral tegmental area (VTA) (Guiard et al. 2008). These neurons are anatomically and functionally interconnected and send reciprocal interactions and projections to the limbic system and cortex (Guiard et al. 2008). Therefore, the monoaminergic system is involved in many behavioural symptoms including mood, vigilance, motivation, fatigue, and psychomotor retardation or agitation (Chopra et al. 2011). In addition, antidepressant pharmacological therapies are based in modulating the

monoaminergic system. The inhibition of the MAO prevents the monoamine´s degradation. Tricyclic antidepressants block not only 5-HT and NA reuptake but also other neurotransmitters receptors inducing high adverse side effects. Hence, selective reuptake inhibitors have been developed to increase 5-HT or 5-HT and NA (Nutt 2008). Nevertheless, the mechanism of action of antidepressants is not fully understood because depressive patients need several weeks of treatment to the achievement of therapeutic response (Kronenberg et al. 2014). For this reason, new hypotheses have been developed in order to explore alternative pathophysiological mechanisms involved in the neurobiology of depression. The components of this system will be detailed next, except for the involvement of DA that will be explained in section *2.5. Reward circuit and dopaminergic system during depression.*

2.1.1. Serotonin

5-HT is an indolamine which has been directly associated with depression since its implication in mood control (López-Muñoz and Alamo 2009; Artigas 2013). 5-HT is synthesized from tryptophan (TRP) by the action of the enzyme 5-hydroxytryptophan and the level of 5-HT synthesis varies with the TRP availability (Schaechter and Wurtman 1990). Serotonergic neurons project from the raphe nuclei to the cerebral cortex, hypothalamus, thalamus, basal ganglia, septum and hippocampus (HC) (Hamon et al. 1990). The serotonergic pathway is involved in the regulation of sleep, appetite, body temperature, metabolism, and libido (Hamon et al. 1990). Several studies demonstrated that acute TRP depletion induced decrease in mood in remitted drug-free depressive patients and in healthy controls with a family history of depression (Ruhé et al. 2007). Post-mortem and preclinical studies, using positron emission tomography, examined the 5-HT receptors 1A, 2A, 1B and found a

serotonergic dysfunction in depression (Köhler et al. 2015). Moreover, the serotonin 1A receptor (5-HTA1) seems to play an important role in depression and anxiety disorders (Köhler et al. 2015). Interestingly, stress can modulate the serotonergic pathway. In this sense, acute stress increases the release of 5-HT (Lanfumey et al. 2008), whereas chronic stress induces a decrease in the 5-HT activity and a depletion of 5-HT stores (McKittrick et al. 2000). In section *2.6. Inflammatory reactions and oxidative stress in the pathophysiology of depression*, the metabolism of 5-HT from tryptophan will be described.

2.1.2. Noradrenaline

NA is implicated in the control of arousal, attention and stress responses (Weinshenker and Holmes 2015). L-tyrosine is the precursor of NA and DA and its availability influences the rates of these neurotransmitters, with functional consequences (Fernstrom and Fernstrom 2007) (See Fig. 6, section *1.1. Metabolism of dopamine*). Noradrenergic neurons are located in the locus coeruleus and project to different brain areas including the hypothalamus, basal ganglia, limbic system, and cerebral cortex (Samuels and Szabadi 2008). Noradrenergic system has been associated with sleep, memory, learning and emotions (Pasquini et al. 2014). Various studies showed that acute L-tyrosine depletion induced mood changes in depressive patients (Delgado and Moreno 2000; Ruhé et al. 2007). Moreover, numerous alterations in the noradrenergic system have been shown in depressive patients and in postmortem brains when compared with healthy controls (Delgado and Moreno 2000; Moret and Briley 2011) including decreased concentrations of NA and its metabolite (3-methoxy-4-hydroxyphenylglycol) in the cerebrospinal fluid (Roy et al. 1986; Gudmundsson et al. 2007).

2.2. Other neurotransmitters involved in the control of mood

Additional neurotransmitter systems, apart from the monoaminergic system, have been studied to elucidate their implication in the pathophysiology of emotional disorders including glutamate (Mathews et al. 2012) or nitric oxide (Dhir and Kulkarni 2011).

In this sense, glutamate is the major excitatory neurotransmitter and is extensively dispersed in the brain (Mathews et al. 2012). Growing evidence shows that glutamate plays an important role in regulating neuroplasticity, learning and memory (Malenka and Nicoll 1999). Essential glutamatergic system regulation is needed in order to prevent excitotoxicity (Mathews et al. 2012), which is involved in several nervous system disorders such as Alzheimer´s disease (Francis 2003). Several evidences from imaging and post-mortem studies as well as experimental studies indicate the involvement of the glutamatergic system in the pathophysiology of emotional disorders including depression (Mathews et al. 2012; González-Sepúlveda et al. 2015). Moreover, chronic stress induces excessive glutamatergic neurotransmission inducing dendritic retraction and loss of spines in brain regions associated with depression such as prefrontal cortex (PFC), HC and amygdala (Gorman and Docherty 2010).

In addition, nitric oxide is a neurotransmitter involved in different physiological mechanisms. Concretely, in the brain, nitric oxide has been implicated in neurogenesis, synaptic plasticity, modulating sexual and aggressive behaviours as well as learning and perception of pain (Esplugues 2002). The precursor of nitric oxide is L-arginine, and it is produced by the action of the enzyme nitric oxide synthase (Knowles and Moncada 1994). In the brain, this enzyme is localized in the HC, dorsal

raphe, and locus coeruleus (McLeod et al. 2001). There are several evidences that have shown a role of nitric oxide in depression (Dhir and Kulkarni 2011). Suzuki and colleges (Suzuki et al. 2001) found that the levels of nitric oxide were altered in most depressive patients. Pre-clinical studies have suggested that the decrease or the block of the synthesis of nitric oxide in the brain have antidepressant-like effects (Dhir and Kulkarni 2011). Moreover inhibitors of nitric oxide synthase increased the extracellular levels of 5-HT and DA in the rat HC (Dhir and Kulkarni 2011).

2.3. Hypothalamic-Pituitary-Adrenal axis and depression

The hypothalamic-pituitary-adrenal axis (HPA) axis is considered the main physiological system in the control of stress. It is activated by a wide range of psychological experiences and physiological perturbations (Armario 2010). Hence, physical or psychological signals of stress are finally integrated in cortical areas of the brain (McEwen 2007; Belmaker and Agam 2008) and appropriated inputs are transmitted to the paraventricular nucleus of the hypothalamus inducing the release of corticotropin-releasing factor (CRF). CRF stimulates the anterior pituitary producing the release of adrenocorticotropic hormone (ACTH) to the bloodstream. ACTH acts in the adrenal glands activating the secretion of glucocorticoids; cortisol in humans and corticosterone in experimental animals (Herman et al. 2003; Belmaker and Agam 2008). The release of glucocorticoids induces a series of metabolic effects proposed to reduce the detrimental effects of stress and maintain homeostasis (Belmaker and Agam 2008) by negative feedback to the hypothalamus and the anterior pituitary. This mechanism of control produces a decrease in CRF and ACTH and consequently, reduces blood levels of glucocorticoids (Herman et al. 2003) (Fig. 1).

The amygdala exerts excitatory control over the hypothalamus to stimulate the HPA axis, which, via increased cortisol levels, acts in a positive feedback manner to further stimulate the amygdala (Villanueva 2013). On the contrary, the HC exerts inhibitory control over the HPA axis through the glucocorticoid receptors. This negative feedback mechanism is critical for controlling the activity of the HPA axis (Jacobson and Sapolsky 1991; Swaab et al. 2005). The PFC is also involved in the negative feedback over the HPA axis improving the control of this axis (Herman et al. 2005).

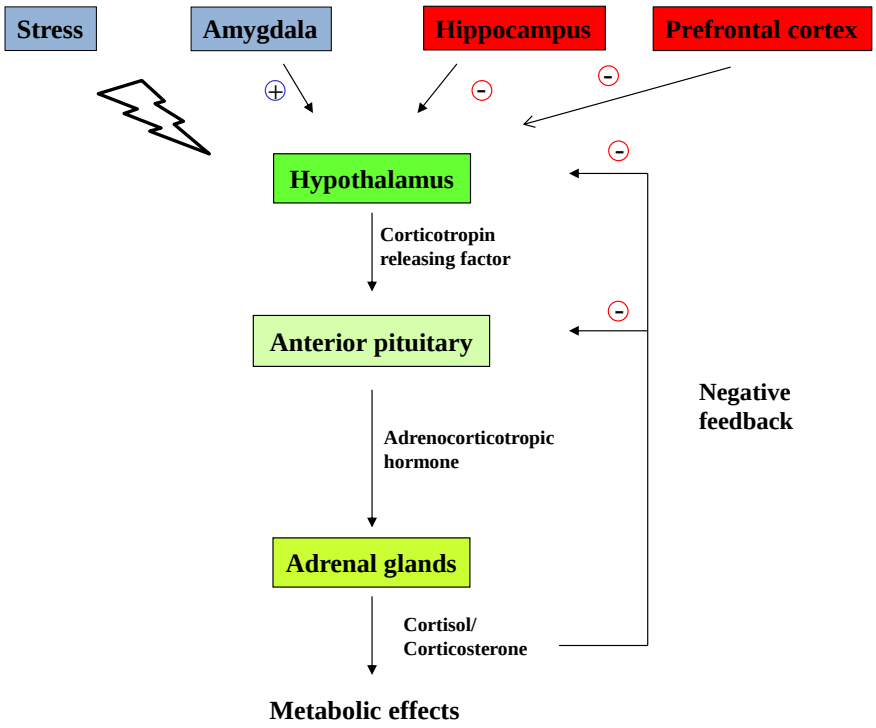


Figure 1. Schematic representation of hipothalamic-pituitary-adrenal axis

Several clinical and basic studies postulate that half of depressive patients present a maladaptive response to stress (Schneiderman et al. 2005; Kanter et al. 2008) due to dysfunctions of the HPA axis (Krishnan and Nestler 2010; Villanueva 2013). Concretely, depression is associated with

hyperactivity of the HPA axis as a consequence of impaired negative feedback mechanism (Kern et al. 2012). Hence, HPA axis is continuously activated and high levels of glucocorticoids are secreted (Chrousos and Kino 2007). Depressive patients show impaired glucocorticoid-receptor-mediated negative feedback (Brown et al. 2004), adrenal hyperresponsiveness to circulating ACTH (Parker et al. 2003) and hypersecretion of CRF (Nemeroff and Owens 2002). Indeed, some assays to evaluate HPA dysfunction such as the dexamethasone suppression test and the dexamethasone/corticotropin-releasing test have been helpful in the diagnosis of depression and to prognosticate the response to antidepressant treatment. Therefore, the administration of dexamethasone, a potent synthetic glucocorticoid, suppresses ACTH secretion by inhibiting the negative feedback at the hypothalamic and anterior pituitary level (Rush et al. 1996; Villanueva 2013). Consequently, about half of the most severely depressive patients show a deregulated cortisol-suppression response in the dexamethasone test (Carroll et al. 2007; Belmaker and Agam 2008). In addition, the administration of glucocorticoids to experimental animals induced depression-like behaviour (Johnson et al. 2006). Indeed, the excess of glucocorticoids may reduce the neurogenesis in the HC (See section *2.4. Neuronal plasticity in the pathophysiology of depression*) and produce atrophic changes in hippocampal subregions due to neurotoxic factors (McEwen 2007). These modifications could contribute to the hippocampal volume reduction seen in depression (Nestler et al. 2002) and to the hippocampal-dependent-memory impairments in animals and humans or verbal declarative memory alterations in depressive patients (de Quervain et al. 2000; Willner et al. 2013). Interestingly, this hyperactivation of the HPA axis may also damage the amygdala and the PFC as well as the HC, whose volumes are decreased in chronic stress situations and depression (Nestler et al. 2002).

2.4. Neuronal plasticity in the pathophysiology of depression

Neuronal plasticity refers to the ability of the central nervous system (CNS) to perceive, respond and adapt to environmental (external and internal) stimuli (Cattaneo et al. 2015). This mechanism involves neuronal remodeling, formation of novel synapses and birth of new neurons (Calabrese et al. 2014; Cattaneo et al. 2015). Neural plasticity impairments are associated with psychiatric disorders as depression, autism or drug addiction (Calabrese et al. 2014; Cattaneo et al. 2015; Wang et al. 2015) and involve neurotrophic factors such as the brain-derived neurotrophic factor (BDNF) and neurogenesis.

Neurotrophic factors play an important role in supporting neuronal survival and modulate the critical steps of network formation during brain development (Calabrese et al. 2014). In addition, neurotrophic factors are crucial intermediaries of neural plasticity also in adulthood, controlling axonal and dendritic growth and remodeling, membrane receptor trafficking, neurotransmitter release as well as synapse formation and function (Lu et al. 2005a).

BDNF is critical for neuronal plasticity (Belmaker and Agam 2008), enhances neurogenesis (Wang et al. 2015) and is also involved in memory processes. Moreover, various studies have confirmed that, in depressive patients, the expression of BDNF is reduced in brain areas, such as the HC and the PFC (Tsankova et al. 2006; Pittenger and Duman 2008; Cattaneo et al. 2015). Preclinical studies have demonstrated the relation between stress and BDNF, since chronic exposure to different stress paradigms in animal models such as chronic mild stress, social defeat or maternal separation induces a reduction of this neurotrophic factor (Tsankova et al. 2006; Pittenger and Duman 2008; Aso et al. 2008; Aso et al. 2009;

Martini et al. 2014; Cattaneo et al. 2015). Several experimental studies have exposed that antidepressant drugs can modulate not only BDNF expression but also other neurotrophic factors (Berton and Nestler 2006; Castrén and Rantamäki 2010). This hypothesis is in agreement with clinical data showing that BDNF serum levels, which are reduced in depressive subjects, can be normalized only in patients that exhibited responses to the pharmacological intervention (Yoshida et al. 2012; Molendijk et al. 2014). Nevertheless, other factors such as members of the insulin-like growth factor, fibroblast growth factor and vascular endothelial growth factor families are also associated with depression (Castrén et al. 2007). These growth factors appear to be involved in adult hippocampal neurogenesis, atrophic changes, and synaptic plasticity of hippocampal neurons (Pittenger and Duman 2008; Krishnan and Nestler 2010). However, some studies show contradictory data. Therefore, pre-clinical experiments have found that increasing BDNF function in the nucleus accumbens (NAc) and amygdala has disruptive effects on measures of anhedonia, anxiety, and social interaction (Krishnan and Nestler 2008; Krishnan and Nestler 2010).

In addition to neurotrophic factors, neurogenesis is also involved in the pathophysiology of depression (Krishnan and Nestler 2010; Kern et al. 2012). Neurogenesis has been defined as the process in which newborn neurons are generated from progenitors to functionally integrate in the neuronal network (Ming and Song 2005; Aimone et al. 2014). At adulthood, neurogenesis mainly occurs in two regions: in the sub-ventricular zone, and then neurons migrate into the olfactory bulb to convert in interneurons and also in the sub-granular zone of the dentate gyrus (DG) of the HC, where new granule neurons are continually generated (Calabrese et al. 2014). Neurogenesis in the HC could be involved in enhancing neural plasticity. This mechanism seems to be

critical for spatial learning, pattern discrimination, contextual memory and mood regulation, behaviours which are altered in depression (Clelland et al. 2009; Cattaneo et al. 2015). In addition, stress can produce detrimental changes in hippocampal neurogenesis (Belmaker and Agam 2008) due to an excess of glucocorticoids (McEwen 2007). Various preclinical studies have demonstrated that most antidepressants and environmental interventions with antidepressant-like effects stimulate adult hippocampal neurogenesis (Sahay and Hen 2007).

2.5. Reward circuit and dopaminergic system during depression

The DA system is associated with the rewarding effects of natural stimulus like food, sex and social interaction, but also drugs of abuse (Koob and Le Moal 2001; Nestler and Carlezon 2006). This circuit projects from the VTA to limbic nucleus such as NAc and amygdala, as well as the PFC and the HC (Russo and Nestler 2013). This circuit will be described in detail in section *1. Dopaminergic system in drug abuse*, regarding its crucial role in the neurobiology of addictive phenomenon, in particular related to cocaine addictive effects.

In addition, different studies have described the role of rewarding circuits in the pathophysiology of depression since this disorder is associated with anhedonia and lack of motivation for plesurable activities as described in the DMS-5 (American Psychiatric Association 2013). Therefore it has been proposed that the DA system contributes to the pathophysiology of depression (Nestler et al. 2002). A defective DA system in the NAc might be associated with a dysfunctional response to normal rewards. Moreover, a dysfunction of DA in the PFC is related to loss of mental energy, loss of drive, and fatigue (Nutt 2008).

Some reports have shown that stress, in animal models of depression, strongly triggers the DA system (Nestler and Carlezon 2006). It has been proposed that the activation of the VTA by stress represents a positive coping mechanism by increasing an individual's motivation and drive to manage actively with the threat (Everitt and Wolf 2002; Krishnan and Nestler 2010). Nevertheless, it has been suggested that chronic exposure to stress induces neuroadaptations in the VTA-NAc pathway contributing to other behavioural alterations related to depression (Russo and Nestler 2013). In addition, several studies have exposed that treatment with antidepressant may modify dopaminergic activity in the VTA, and that changes in dopaminergic transmission pathway can modulate depression-like behaviour in animal models (Russo and Nestler 2013). Other studies in rodents demonstrated a link between DA system and BDNF, since this factor controls the expression of D3 receptor gene (Sokoloff et al. 2002). These results suggest the neurotrophic hypothesis of depression implicates also the reward circuit (Dailly et al. 2004). Furthermore, symptoms of psychomotor retardation are probably regulated by dysfunctional dopaminergic transmission in depressive patients (Flint et al. 1993; Prins et al. 2011).

2.6. Inflammatory reactions and oxidative stress in the pathophysiology of depression

Clinical and experimental data suggest that the pathophysiology of several neuropsychiatric disorders involves the activation of the immune system in response to inflammatory agents. Experimental studies show that the administration of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), elicits sickness behaviour and depressive symptoms such as decreased in general activity, reduced food intake, social withdrawal, and cognitive alterations in rodents (Dantzer et al. 2008). Similarly, in clinical studies, the administration of inflammatory

agents in healthy humans (Reichenberg et al. 2001; Brydon et al. 2008) produces clear symptoms related to depression (including fatigue, anhedonia, anorexia, poor sleep, and others). In addition, other clinical studies report in depressive patients increased plasma and cerebrospinal fluid levels of pro-inflammatory cytokines as interleukin-6 (IL-6), IL-1β and TNF-α (Miller et al. 2009; Dantzer et al. 2011; Anderson et al. 2014), as well as acute phase proteins, chemokines, adhesion molecules, and inflammatory mediators (Miller et al. 2009). Moreover, depressive patients with antidepressant resistance display higher plasma levels of several pro-inflammatory cytokines and c-reactive protein when compared with treatment-responder patients (Chopra et al. 2011; Cattaneo et al. 2015). In summary, cytokines have been demonstrated to access the CNS and act in brain areas associated with the pathophysiology of depression (Miller et al. 2009). Nevertheless, it is not well understood if the activation of inflammatory pathways in the CNS during depression occurs first at the periphery or if stress or other insult cause inflammatory activation in the brain (Dantzer et al. 2008; Miller et al. 2009). Recent reviews report that the releasing of these pro-inflammatory mediators has been produced by the pathophysiological effects of stress (Myint et al. 2007; Miller et al. 2009) inducing the activation of the immune response not only in the periphery but also in the CNS (Miller et al. 2009; Réus et al. 2015a). The immune response in the CNS is controlled by microglia and astrocytes (McNally et al. 2008). However, preclinical studies have shown that under chronic stress, microglia can become a source of pro-inflammatory cytokines (Dantzer et al. 2011; Réus et al. 2015a) contributing to the development of depression.

As mentioned above, the production of pro-inflammatory cytokines contributes to the pathophysiology of depression by altering neuroendocrine, synaptic plasticity and neurotransmitter function (Chopra

et al. 2011). In this sense, pro-inflammatory cytokines are involved in the deregulation of the HPA axis producing glucocorticoids resistance and increasing the baseline activity of the axis (Maes et al. 1993; Anderson et al. 2014) and they promote abnormalities in neural plasticity, including a decrease of the neurotrophic support and impaired neurogenesis in brain areas relevant in behaviour and cognition (Miller et al. 2009). In addition, as explained in Gracia-Rubio et al. 2015, clinical and preclinical studies suggest that pro-inflammatory cytokines alter TRP metabolism within the brain, modifying the activity of 5-HT neurotransmitter system (Miller et al. 2009; Christmas et al. 2011). Consequently, the metabolic TRP route becomes deregulated during depression, increasing kynurenine (KYN) synthesis and enhancing the alternative TRP metabolic pathway by activating indoleamine 2,3-dioxygenase (Christmas et al. 2011) (see Fig. 2), reducing the availability of TRP to be metabolized in 5-HT. Interestingly, metabolites of the TRP-KYN pathway may regulate the brain homeostasis as well as modulate other different neurotransmitter systems including glutamate and DA (Miller et al. 2009; Myint 2012). In fact, kynurenic acid, an intermediate metabolic product of the TRP-KYN pathway behaves as a N-methyl-D-aspartate (NMDA) antagonist, displaying neuroprotective actions in the brain and inhibiting the release of the excitatory neurotransmitter glutamate as well as the release of DA in discrete brain areas (Borland and Michael 2004; Sas et al. 2007; Klein et al. 2013). On the other hand, quinolinic acid, one of the pathway's final products, seems to act as an NMDA agonist, promoting glutamate release and contributing to excitotoxicity and oxidative stress in the brain (Müller and Schwarz 2007; McNally et al. 2008). Similarly, 3-hydroxykynurenine also displays neurotoxic effects by promoting the formation of reactive oxygen species (ROS) and causing neuronal apoptosis (Okuda et al. 1998; Stone 2001). The importance of the alteration in the balance between kynurenic acid and quinolenic acid (neuroprotective vs. neurotoxic) in the

brain of depressive patients, has been suggested to be related to the pathophysiology of depression (Myint and Kim 2003; Chopra et al. 2011). Furthermore, the imbalance of this metabolic TRP route induces a detrimental 5-HT synthesis that has been directly associated to the development of depressive symptoms in humans and in experimental animal models (Laugeray et al. 2010; Gabbay et al. 2010; Steiner et al. 2011).

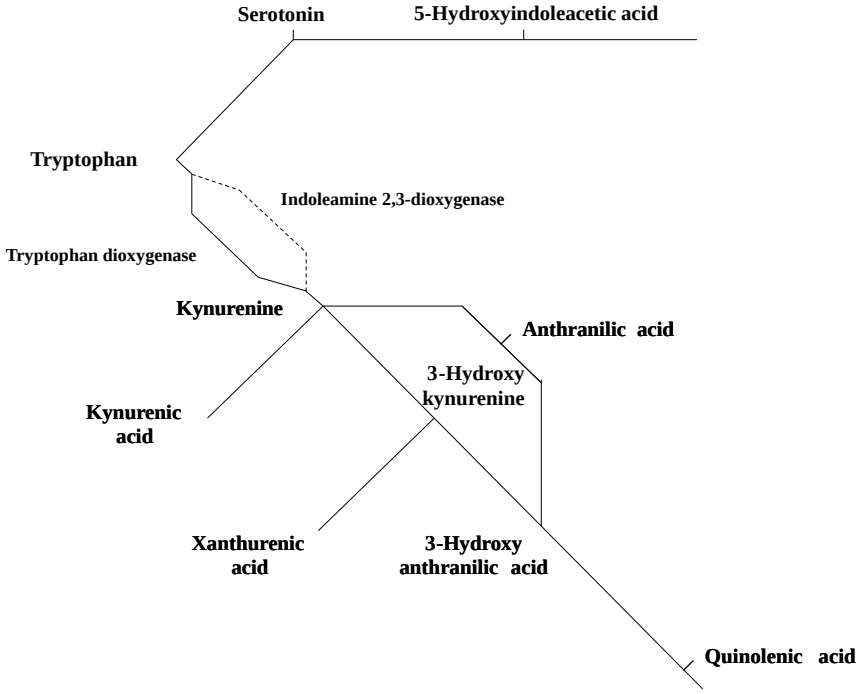


Figure 2. Schematic representation of the tryptophan-kynurenine metabolic pathway (adapted from Christmas et al. 2011).

In addition, oxidative stress is also involved in the pathophysiology of depression. Oxygen reactivity contributes in the transference of high-energy electrons, and hence participates in the generation of adenosine-5-triphosphate (ATP) through oxidative phosphorylation (Burton and Jauniaux 2011). Furthermore, ROS act as second messengers in

intracellular signaling cascades in order to maintain cells´ homeostasis (Dröge 2002). Oxidative stress appears when the generation of ROS exceeds the antioxidant mechanisms (Burton and Jauniaux 2011) and it has been implicated in the pathogenesis of several by causing neuronal damage (Chopra et al. 2011). ROS induce lipid peroxidation, protein carbonylation and DNA damage (Leonard and Maes 2012) and decrease the efficacy of glutathione peroxidase, catalase and superoxide dismutase, that are involved in antioxidant mechanisms (Bilici et al. 2001; Chopra et al. 2011). As mentioned above, depression compromises the activation of the immune system by increasing the release of pro-inflammatory cytokines that induces ROS (Miller et al. 2009) as well as psychological stress which may increase lipid peroxidation (Bilici et al. 2001). Moreover, human studies have found that several markers of oxidative stress are increased in depressive patients vs. controls (Maes et al. 2009; Anderson et al. 2014).

3. Vulnerability factors influencing the development of depression

Depression is thought to comprise a heterogeneous group of diseases caused by genetic, epigenetic and environmental factors (Nestler 2014). Moreover, experimental (Palanza et al. 2001; Renoir et al. 2011) and clinical (Kessler 2003; Altemus 2006) data show that sex or gender factors are involved in the vulnerability of depression since the risk of suffering depression is twofold higher in woman than in men (Weissman et al. 1996; Kudielka and Kirschbaum 2005; Sherin and Nemeroff 2011).

3.1. Genetic factors involved in depression

Genetic factors are involved in the development of depression, as presented by twin, family and adoption studies (Lohoff 2010; Smoller 2015). Actually, twin studies have indicated that depression is associated with genetic factors, with a heritability of around 40% (Kendler et al. 2005; Smoller 2015) and family studies have shown about two to threefold increase in lifetime risk of developing depression among first-degree relatives (Lohoff 2010). However, adoption studies, which provide an alternative line to separate genetic and environmental factors, have found controversial results (Lohoff 2010). Several studies have suggested a twofold increase risk of developing depression in the biological relatives (Cadoret et al. 1985), whereas other studies have found no difference in the rate of mood disorders (von Knorring 1983). In addition, family and twin studies have evaluated subtypes of depression that may induce higher familial risk (Smoller 2015). Particularly, higher familial recurrence risk and heritability have been related to earlier-onset and repeated episodes of depression (Kendler et al. 2005; Smoller 2015) in addition to increased depression severity (Klein et al. 2002; Smoller 2015), although these results have not been observed in all studies developed.

Therefore, other studies have been developed to elucidate the involvement of genetic factors in depression such as linkage and gene candidate studies. In this term, linkage studies are based in evaluating chromosomal fragments that might present vulnerability genes that are inherited with an illness with higher probability than expected by chance in families. Although some linkage studies in depression have proposed various regions in the genome that might show risk alleles, the results obtained have been discrepant, consequently, no established universal genetic risk factor or causative gene for depression has been recognized. These results

highlight that depression is a complex disorder with a complicated mode of inheritance, in which several genes with small effects are involved, and identifying genetic factors is complicated by an important gene–environment component. For further information about linkage studies´ results see review (Lohoff 2010).

In addition, several studies were focused in evaluating candidate genes in neurotransmitter, neuropeptide, and neuroendocrine systems associated with antidepressant targets or implanted in animal models (Smoller 2015). Almost 200 genes have been tested by several groups. However, the results are incongruent between them (Flint and Kendler 2014; Smoller 2015). Flint and Kendler (2014) analyzed by meta-analysis the candidate genes searching articles until 2013 and summarized the results obtained for 26 genes. It is important to note that in this study only seven genes yield a significant ($p<0.05$) result. These results comprised genes involved in the serotonergic system including its transporter 5HTTP/SLC6A4 (López-León et al. 2008; Clarke et al. 2010), and one of its receptors HTR1A (López-León et al. 2008; Kishi et al. 2012). In the dopaminergic system is also involved its transporter DAT/SLC6A3 and the dopamine 4 receptor (López-León et al. 2008). Moreover, there are genes associated with metabolic pathways such as APOE (López-León et al. 2008), GNB3 (López-León et al. 2008) and MTHFR (Lewis et al. 2006; López-León et al. 2008). The disadvantage of this kind of studies is that genes evaluated are first pre-selected; therefore the involvement of other genes may be overlooked.

Finally, to improve the candidate gene studies, a new method called Genome Wide association studies (GWAS) was developed (Hindorff et al. 2009). GWAS are able to genotype 500,000 to 1 million single nucleotide polymorphisms across the genome in cases and healthy controls. GWAS

projects have the benefit that no genes are preselected, therefore this technique could show new pathways implicated in emotional disorders that were not previously considered (Pearson and Manolio 2008; Lohoff 2010). In the review of Flint and Kendler (2014), the nine published GWAS were summarized for depression although no robust results were found. However, Kohli and colleagues (Kohli et al. 2011) found a genome-wide significant association on chromosome 12 associated to neuron-specific neutral amino acid transporter (SLC6A15). Nonetheless, and as a result of technical complications, lack of evidence may sometimes be the result of troubles in detecting an effect (Lein et al. 2007). Moreover, GWAS data can be used to further constrain the likely genetic architecture of depression, by using marker results that do not reach genome-wide significance. This is important because it might be that the genetic architecture of depression consists primarily of rare but relatively large effect loci (Flint and Kendler 2014).

3.2. Epigenetic factors participating in depression

Depression results from a complex interplay of vulnerability genes and environmental factors that act cumulatively throughout the individual's lifetime. Among these environmental factors, stressful life experiences, particularly early in life, have been proposed to induce a critical effect on brain development, causing permanent changes that may confer vulnerability for mental health outcomes along lifespan (Lopizzo et al. 2015), although stress can also produce negative alterations during adulthood. Therefore, exposure to detrimental environmental situations produces stable changes in gene expression, neural circuit function, and finally behaviour. Increasing evidence indicates that these sustained abnormalities are maintained by epigenetic modifications in specific brain regions associated with depression that consist in altering chromatin structure without modification of DNA sequence (Nestler 2014; Nestler et

al. 2015). Hence, epigenetic mechanisms control the spacing of nucleosomes and the degree of condensation determining gene activity (Nestler et al. 2015). The most important biochemical processes that control epigenetic mechanisms include modification of histones such as acetylation, ubiquitination or methylation as well as DNA methylation (Nestler et al. 2015). DNA methylation induces more stable epigenetic changes than histone modification which are considered, in general, reversible (Cedar and Bergman 2009). Nestler and colleagues (Nestler et al. 2015) summarize the principal studies developed to study epigenetic mechanisms in depression, most of which have been assessed in animal models exposed to stress such as social defeat, maternal stress or maternal separation, although some of them are performed in depressive patients. In this review the epigenetic mechanisms induced by antidepressant treatments were also included. Within the epigenetic alterations found in the limbic system, there are genes related to neurotrophic factors such as BDNF (Tsankova et al. 2006), involved in the HPA axis including Nr3C1 (McGowan et al. 2011); related to glutamatergic transmission as for example Gad1 (Zhang et al. 2010); associated with dendritic spines as Rac1 (Golden et al. 2013) or implicated in the release of neurotransmitters including SYN1 (Cruceanu et al. 2013), among other genes. It is important to note that some of these epigenetic alterations can be reversed by antidepressant treatment suggesting a new field of knowledge to develop new therapies (Nestler 2014).

3.3. Sex and gender factors associated to depression

The prevalence of depression is twofold higher in women than men (Weissman et al. 1996; Kudielka and Kirschbaum 2005; Sherin and Nemeroff 2011). These differences have been found across many nations, cultures, and ethnicities evaluated (Weissman et al. 1996). Clinical studies

have shown that depressive disorders are infrequent during childhood and show no gender differences (Angold and Rutter 2008). However, prevalence seems to rise in late adolescence and early adulthood, particularly in females (Klein et al. 2002). Puberty may thus be related to the emergence of sex differences (Cohen et al. 1993), because of the involvement of biological alterations and social transitions (Fombonne 2001). Therefore, by late adolescence, the depression ratio between girls and boys is 2:1, respectively, and this gender ratio is maintained throughout adulthood (Nolen-Hoeksema 2001). Nevertheless, at ages older than 65 years, both men and women show a decline in depression rates, and the prevalence becomes similar (Albert 2015). Some studies support higher rates of first-onset depression in females rather than a great number or longer duration, of episodes (Kessler et al. 1993); whereas others studies show female preponderance in recurrent chronic depression (Piccinelli and Wilkinson 2000).

In order to explain the reasons behind gender differences, biological, psychosocial and sociocultural factors must be considered. Regarding biological factors, various studies reported no genetic influence in gender differences (McGuffin et al. 1996; Piccinelli and Wilkinson 2000). However, more recent findings indicate that the heritability of depression is higher in women than in men (Kendler et al. 2005; Kendler and Gardner 2014). In addition, changes in reproductive hormones in utero including estradiol and progesterone, during puberty, the estrous cycle, pregnancy, and menopause modify brain structure and function, and these hormones are suggested to be involved in the increased prevalence of affective disorders in women. In the luteal phase of the menstrual cycle, HPA axis responsiveness increases and glucocorticoid feedback sensitivity decreases, destabilizing these homeostatic systems in vulnerable women (Altemus 2006). Moreover, animal studies show a significant difference

between sexes in depression-like behaviour paradigms indicating a higher sensitivity in females when compared with males (Palanza et al. 2001; Renoir et al. 2011). In addition, a reduction in behavioural levels of anxiety during proestrous phase has been shown, when levels of ovarian steroids hormones are higher (Butcher et al. 1974; Martini et al. 2014). Consequently, sex differences in emotional disorders and hormonal modulation of them are expected to provide an important window into the pathophysiology of anxiety and depression (Altemus 2006). However, the mechanisms are not fully understood; therefore, specific treatments to women have not been yet developed (Albert 2015). Concerning psychosocial factors, several recent studies have shown that women are more likely than men to develop depression in response to interpersonal stressors (Nolen-Hoeksema et al. 1999) since women have stronger affiliated feelings than male; subsequently they need greater social support for their psychological health (Piccinelli and Wilkinson 2000). Moreover, during adolescence and adulthood women appear to be more likely than men to respond to stress and distress with rumination, focusing inward on feelings of distress and personal worries rather than taking action to remove their distress, whereas men tend to distract themselves from their mood by engaging in physical or instrumental activities (Piccinelli and Wilkinson 2000). Several experimental studies have shown that people who ruminate in response to stress have an increase risk to develop depressive symptoms (Nolen-Hoeksema et al. 1999). In relation to sociocultural features, women face a number of strains that could contribute to their higher rates of depression (Nolen-Hoeksema et al. 1999). Women often have full-time paid jobs and also do nearly all the child care, housework, and care for sick and elderly family members. This role causes a general distress, inducing depressive symptoms (Nolen-Hoeksema 2001). Finally, women are more likely to report physical and

psychological symptoms and to seek medical help (Piccinelli and Wilkinson 2000).

3.4. Environmental factors influencing depression

As mentioned before, a variety of stressful life events may increase risk for developing depression (Kessler 1997; Smoller 2015) including poverty (Hackman et al. 2010), negative family relationships (Kolb and Gibb 2011), early life stress (Widom et al. 2007; Bhatia and Bhatia 2007), and traumatic events (Krishnan and Nestler 2008). There appears to be a complex relationship among stressful situations, our mind and body's reaction to stress, and the onset of clinical depression (Krishnan and Nestler 2008). It has been described that these stressful life situations induce alterations in neurobiological factors, explained above, such as alteration of HPA axis (Krishnan and Nestler 2010) or release of pro-inflammatory cytokines (Miller et al. 2009), as well as epigenetic changes (Nestler 2014; Nestler et al. 2015). In addition, there are other factors that confer vulnerability to depression such as comorbidity with other psychiatric disorders, including substance use disorders (Cheetham et al. 2010) or other medical illness including cardiovascular diseases, respiratory disorders, cancer or several neurological conditions (Parkinson´s disease or stroke) (Katon 2003). Indeed, early life stress such as child abuse or neglect occurring during vulnerable stages of development is one of the major means whereby the environment influences the development of depression (Saveanu and Nemeroff 2012). Furthermore, there is increasing evidence that suggests an elevated comorbidity between depression and drug addiction, mainly in people who suffered negative experiences during childhood (Dube et al. 2003; Gerra et al. 2009). This thesis will focus on early life stress (See *Early life experiences* section) and substance use disorders (See *Drugs of abuse and*

substance use disorders section); therefore they will be described in detail in the following sections.

Early life experiences on the control of mood

1. Brain development

Brain development will be detailed in order to understand the involvement of early life experiences on behavioural modulation.

In humans, brain development begins in the 3rd gestation week (GW) and continues into early adulthood, probably along the lifespan (Stiles and Jernigan 2010). This process can be divided in different stages; the prenatal period that is genetically determined and can be modulated by maternal environment. The postnatal period, in which the interaction of genetic information with a wide set of environmental exposures and experiences determines the brain development process in this stage (Tau and Peterson 2010; Kolb and Gibb 2011) and the adolescent stage in which brain areas related to emotional control are under maturation process (Crews et al. 2007; Giedd 2008).

1.1. Brain development during prenatal period

The neuro-morphogenesis process in humans begins, as explained before, at GW3 with a range of processes called *gastrulation* (Stiles 2008; Stiles and Jernigan 2010). Then, the neural tube is formed, a process called *neurulation,* and around GW4 to GW12, the rostral portion of the neural tube begins to expand forming the three primary brain vesicles. The most anterior of these embryonic brain vesicles is called the "prosencephalon" which is the precursor of the forebrain. The middle vesicle is the "mesencephalon" which is the predecessor of midbrain structures, and the most posterior is the "rhombencephalon" which will become the hindbrain. By the end of the embryonic period these 3 vesicles will subdivide in 5, making up the secondary brain vesicles. The

prosencephalon divides into the "telencephalon" and the "diencephalon", that subsequent will become, in posterior stages, the cerebral cortex and subcortical structures and the thalamus and hypothalamus, respectively. The rhombencephalon divides into the "metencephalon" and the "myelencephalon" which will form the brainstem and the cerebellum (Fig. 3) (Stiles 2008; Tau and Peterson 2010).

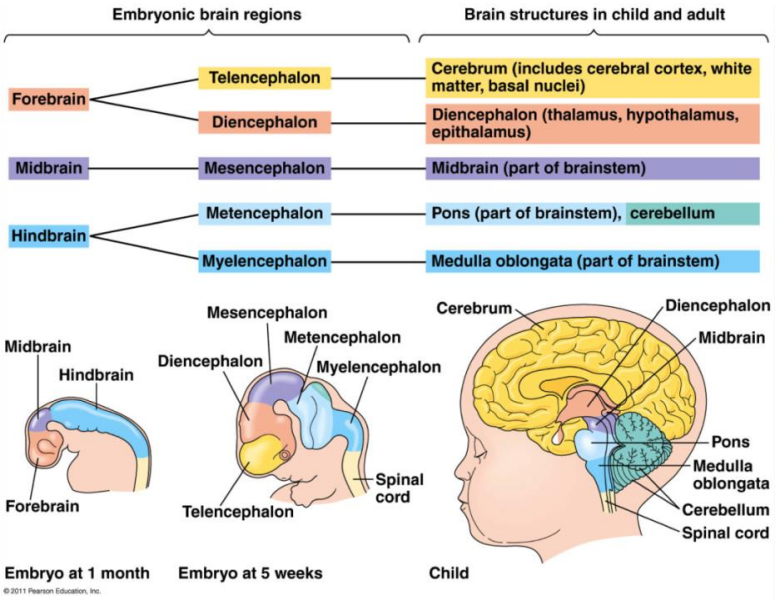


Figure 3. Brain development. From the neural tube to the brain structures which form the child and adult brain (Jane Reece et al. 2011).

After these first weeks of gestation around GW9, begins a new stage characterized by high proliferation of new neurons (Stiles and Jernigan 2010). Furthermore, the principal glial cells, astrocytes and oligodendrocytes, are originated in the ventricular zone (Stiles 2008). These cells play an important role in neural development because of their implication in synapse formation (Clarke and Barres 2013). Microglia is also considered as a kind of glial cell, which is originated from circulating precursors in the blood from the bone-narrow (Jiang and Nardelli 2015).

The role of these cells is especially focused on apoptosis (Jiang and Nardelli 2015) and pruning processes (Schafer et al. 2012).

Neuron proliferation is counterbalanced by the programmed cell death of neurons through a mechanism called apoptosis (Cowan et al. 1984; Stiles and Jernigan 2010). Although neural apoptosis is carried out mainly during the prenatal period, this process also extends until the postnatal period (Stiles and Jernigan 2010). One important function of apoptosis in brain development is regulating the establishment of effective and functional neural circuits (Buss et al. 2006).

When neurons get to their final destination, they start to differentiate into a particular cell type following a process which is influenced by genes and environmental factors. In addition, they need to become integrated into neural networks. This mechanism involves growing dendrites and extending axons that allow them to communicate with other neurons (Stiles and Jernigan 2010; Kolb and Gibb 2011). Neurotransmitters are involved in chemical synapses and are also implicated in cell proliferation, migration, and differentiation (Stiles 2008). However, the expression patterns for neurotransmitters and their receptors change across brain development (Stiles 2008) and mature patterns of neurotransmitters expression are not perceived until adolescence (de Graaf-Peters and Hadders-Algra 2006; Stiles 2008).

1.2. Brain development in the postnatal period

The postnatal period starts at birth and continues mainly through the second decade (Webb et al. 2001), although brain changes occur during lifespan (Stiles and Jernigan 2010).

In the postnatal period also occurs another wave of neurogenesis in the cerebellar cortex, the olfactory bulb, and the DG of the HC (Hatten 1999; Watson et al. 2006). At adolescence, the neurogenesis in the DG starts to decrease, but a small amount continues throughout life (Johnson 2001; Watson et al. 2006). Postnatal neurogenesis in the DG may be guided by hormones, neurotransmitters, growth factors, stress, and environmental cues; moreover these factors could influence learning and memory in adults (Watson et al. 2006).

During this early postnatal period, there is also a huge increase in the growth of axonal and dendritic arborization that involves an excessive synaptogenesis (Goldman-Rakic 1987). Several studies propose that this overproduction may also be the mechanism by which the brain is made ready to receive specific input from the environment (Goldman-Rakic 1987) and it is also thought to maximize the information-carrying capacity of the immature brain (Andersen 2003). Moreover, synapses that make functional connections receive a larger amount of coordinated activity and are stabilized by neurotrophic factors, but the rest of synapses may be eliminated (Tau and Peterson 2010).

The process reducing the density of synapses is called pruning (Huttenlocher and Dabholkar 1997). This mechanism selects the most useful neurons, synapses, and dendrites to optimize the brain functioning, involving the fine-tuning of neuronal circuitry and synapse strengthening in the nervous system (Jiang and Nardelli 2015). Consequently, pruning contributes to more efficient processing of cognition (Andersen 2003) and it is influenced by environmental factors (Jiang and Nardelli 2015).

Myelination also starts at the prenatal period and keeps on throughout childhood and adolescence (Stiles 2008). Hence, white matter increases across most brain regions through childhood, adolescence and adulthood

(Tau and Peterson 2010), although the rate of increase is greatest during childhood (Paus et al. 2001; Matsuzawa et al. 2001). Nevertheless, several studies showed that the majority of cortical regions (grey matter) follow a trajectory of early thickening during early childhood (Shaw et al. 2006; Raznahan et al. 2011), succeeded by a period of decline that begins at late childhood or early adolescence, related to pruning mechanisms (Shaw et al. 2006; Raznahan et al. 2011).

As stated above, in the postnatal period the changes that occur in brain structure and function involve not only genetic elements, but also environmental factors and behavioural responses that stimulate the immature brain to develop the neural circuits (Greenough et al. 1987; Kandel and Squire 2000). Thefore, neural circuits development could follow two bases: experience-expectant systems and experience-dependant systems. In an experience-expectant system, including language or visual skills, development is based on the expectation that suitable environments will supply the information that the brain needs to select the appropriate subset of synaptic connections. Otherwise, in an experience-dependent system, development is unique to each person and most likely involves the active formation of new synaptic connections throughout the lifespan, for example vocabulary (Webb et al. 2001). Moreover, synapses formed from "expecting" experiences are developed in early postnatal period and are found diffusely within the brain. However, synapses formed during later brain development and adulthood, follow the experience-dependant process and they are localized in areas related to processing specific experiences (Greenough et al. 1987; Kolb and Gibb 2011). In addition, glucocorticoids are required for normal brain development because they are implicated in terminal maturation, growth of axons and dendrites and cell survival (Meyer 1983; O' Mahony et al. 2015).

Taking into account the above-mentioned statements, it has been proposed that infancy is a critical period of development, in which rapidly growing structures are more sensitive to damage (Huttenlocher et al. 1984; Trajkovska et al. 2009). Therefore, during this period, detrimental alterations could induce structural and functional impairments in the development of the cerebral cortex (Taylor and Alden 1997; Webb et al. 2001) that may persist until adulthood (Gross and Hen 2004).

1.3. Brain development in the adolescent period

During adolescence, the development of the brain continues with modifications at cellular, molecular and anatomical level (Giedd 2008). Consequently, brain regions involved in cognitive and emotional skills including the PFC, HC, amygdala, NAc, VTA and hypothalamus experience important modifications (Crews et al. 2007). Interestingly, these changes are produced mainly in the PFC and HC (Powell 2006). These brain areas are important as the PFC controls executive functions including cognitive flexibility, memory processes, self-regulation and evaluation of risk and reward (Crews et al. 2007). Whereas, the HC is involved in learning and memory processes (Morris et al. 1982) and the amygdala is associated with the regulation of emotional behaviour (LeDoux 1993). Hence, modifications in these brain regions are related to achievement of adult cognitive and emotional skills (Crews et al. 2007).

Furthermore, magnetic resonance imaging studies have shown that during adolescence starts a decrease of the grey matter (Giedd 2004; Shaw et al. 2006; Powell 2006; Raznahan et al. 2011), probably this mechanism is due to a competitive process guided by environmental experiences in which the most frequently used synapsis are reinforced and the occasionally used synapsis are eliminated by synaptic pruning (Fig. 4) (Giedd 2004; Powell 2006).

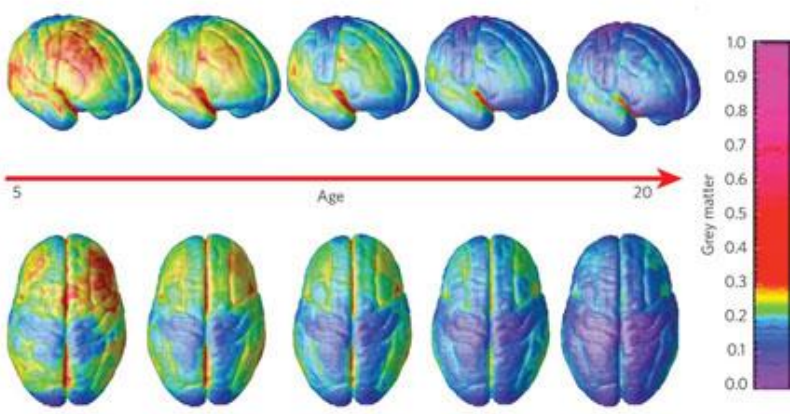


Figure 4. Magnetic resonance images show that gray matter decreases as the brain matures. Red specifies more gray matter and blue, less gray matter (from Powell, 2006).

In addition, several studies state that the peak of neural activity in subcortical regions during adolescence may motivate the increased risk taking and novelty seeking behaviours (Casey et al. 2008). Indeed, experimental studies showed that some rodent neural systems are under maturation during adolescence (Spear 2000). In particular, the maturation of DA neurons in the VTA that project to the PFC and NAc seems to be one of the most critical neural system relevant for processing salient events, including responses to the psychostimulants (Everitt and Wolf 2002; Burke and Miczek 2014). Therefore, in section *3. Implications of early-life detrimental experiences on the development of depression* will be described the relevance of adolescence as a vulnerability period to develop psychiatric disorders.

2. Protective and detrimental factors influencing emotional reactions during childhood

In this thesis project, we have focused on childhood as it is an important period in which environmental experiences play an important role in shaping brain and behaviour (Branchi and Alleva 2006; Lupien et al. 2009; D'Andrea et al. 2010). Moreover, we are interested in psychosocial aspects, including those factors that alter the normal brain development related to early life stress; but also in factors that improve the capacities of children, protective factors. Understanding the causal relationship between early experiences with brain development requires from experimental models; therefore, in this part, we will also refer to experimental models in order to comprehend better some features of these factors that influence the brain development.

2.1. Protective factors during childhood

2.1.1. Positive early experiences: Human evidences

Human studies suggest that exceptional maternal care during postnatal period is related to resilience to psychiatric disorders (Gourion et al. 2008; Korosi 2009). Indeed, clinical programmes try to increase parenting practise in poor and high-risk families in order to improve behavioural and cognitive capacities in children (Hackman et al. 2010). Furthermore, parental aptitude to regulate the emotional state in infants is important to help children to develop strategies for self-regulation (Murray and Cooper 1997b; Tsivos et al. 2015). Intellectual stimulation at home is also important due to the correct development of cognitive skills (Hackman et al. 2010). This stimulation process includes availability of books, computers, trips and parental communication, among others (Hackman et al. 2010) and may explain the decrease in cognitive abilities found in

children with low socio-economic status (Hackman et al. 2010). Interestingly, a warm affection relationship during infancy is related to optimal development and social abilities, including more advanced emotional understanding, higher cognitive and language skills, and less dependence on adults (Belsky and Fearon 2002). Furthermore, developmental theories have stated that peer interaction fosters are involved in different competencies of adult behaviour than those induced by the interplaying with the mother (Branchi and Cirulli 2014). Certainly, throughout facing different types of peer interactions, children acquire knowledge of self-versus others and several social interaction skills such as understanding of the others and role-taking interactions (Branchi and Cirulli 2014).

2.1.2. Positive early experiences: Experimental studies

The study of natural variations in maternal care in rodents has contributed for evaluating the influence of mother-infant interactions within their normal environment (Hackman et al. 2010). Various studies have observed that licking/grooming of pups, a form of tactile stimulation, is significantly different between dams during the postnatal period (Kundakovic and Champagne 2014). Therefore, the adult offspring of dams that exhibit high licking and grooming (High-LG) show reduced behavioural and endocrine responses to stress when compared with the offspring of dams that exhibit low licking and grooming (Low-LG) (Liu et al. 1997; Zhang et al. 2010). Concretely, the offspring of dams that exhibit High-LG display increased glucocorticoid receptors expression in the HC, reduced hypothalamic CRF levels and more modest HPA responses to stress compared with the offspring of dams that exhibit Low-LG (Liu et al. 1997; Zhang and Meaney 2010; Hackman et al. 2010). Moreover, this offspring show increased synaptic density (Liu et al. 2000; Champagne et al. 2008) and a higher capacity for synaptic plasticity in the HC and PFC

(Bredy et al. 2004). Hence these animals show improved learning and memory skills associated with these brain areas (Liu et al. 2000; Bredy et al. 2004; Champagne et al. 2008). Interestingly, cross-fostering studies, in which pups born to High-LG mothers are fostered at birth to Low-LG (and vice versa), indicate a clear relationship between maternal care and the postnatal development of individual differences in behavioural and HPA response to stress (Liu et al. 1997; Zhang and Meaney 2010). Therefore, the rearing mother determines the phenotype of the offspring (Zhang and Meaney 2010). The results of these studies indicate that the behaviour of the mother can "program" stable changes in gene expression that are the basis for differences between individuals in behavioural and neuroendocrine responses to stress in adulthood (Zhang and Meaney 2010). These changes in gene expression are associated with epigenetic mechanisms, and several studies have focused in evaluating the enrolment of maternal behaviour on the epigenetic changes induced in the stress response including the HPA axis and the brain areas implicated in its regulation (Liu et al. 1997; Zhang and Meaney 2010).

Moreover, several studies have been interested in simulating this enhanced tactile experience induced by mothers High-LG, therefore in these experiments infant rodents are given tactile stimulation with a brush several times a day during the postnatal period (Kolb and Gibb 2011; Bale 2015). Adult offspring display not only increased skilled motor performance and spatial learning but also changes in synaptic plasticity (Kolb and Gibb 2010; Bale 2015).

Other alternatively way to intensify sensory and motor capacities is to house animals in complex environments where they can interact with a changing sensory and social environment and to increase motor activity (Nithianantharajah and Hannan 2006; Ros-Simó and Valverde 2012).

These studies have shown a variety of neural changes, including increases in brain size, cortical thickness, neuron size, dendritic branching, spine density, synapses per neuron, glial numbers and complexity, and vascular (Greenough et al. 1987; Kolb and Gibb 2011).

Continuing with this idea, the communal nest (CN) model (Branchi et al. 2006a) provides social enrichment by approximating the natural ecological conditions of mice (Branchi and Alleva 2006). This model consists in a single nest where three mothers keep their pups together and share care-giving behaviour from birth to weaning (Branchi et al. 2006b). The CN provides a highly stimulating environment, allowing the developing pups to interact with its biological and two additional mothers, as well as with a higher number of peers (Hayes 2000). Offspring reared in the CN show enhanced neuronal plasticity including increased level of BDNF and survival of neurons in the HC (Branchi et al. 2006b).

2.2. Detrimental factors during childhood

2.2.1. Adverse early life experiences: Human evidences

Adverse early life conditions in infancy have been associated with brain development alterations (Kaufman et al. 2000) increasing vulnerability to develop a psychiatric disorder throughout life (Lupien et al. 2009; Korosi 2009) such as depression (Widom et al. 2007; Bhatia and Bhatia 2007) or substance use disorder (Dube et al. 2003; Gerra et al. 2009) and inducing cognitive impairments (Korosi 2009).

Parent-child relationship is critical during the brain development (Kolb and Gibb 2011) and differences in early maternal-infant interactions can induce long-term brain developmental effects that will persist into adulthood (Myers et al. 1989; Kolb and Gibb 2011) including parental lost

during childhood (Weller et al. 1991). Maternal-infant relationships can be disrupted by poverty, substance use by the mother or maternal depression, situations where, although the mother figure is still present, her behaviour is aberrant inducing chronic stress in her children (Korosi 2009).

Children and adolescents from low socio-economic status display higher rates of depression, anxiety, attention and conduct disorders (Duncan et al. 1994; Hackman et al. 2010). Moreover, malnutrition or famine related to poverty, natural disaster or war, not only decrease brain size in infancy and produce several adverse effects on neural circuit development but also are associated with elevated risk for psychiatric disorders (Tau and Peterson 2010). These children are exposed to a decrease parental involvement due to greater irritability, depressive, and anxious feelings (Hackman et al. 2010). Parental stress induces discordant discipline, less sensitivity to the needs of the child, reduced verbal communication and insecure attachment to the primary caregiver (Hackman et al. 2010). Several clinical studies suggest that depression in parents may be associated with the development of cognitive and emotional alterations and is related to later psychopathology and atypical development in the offspring (Murray and Cooper 1997a; Tsivos et al. 2015). Parental ability to regulate infant´s emotional state plays a key role in helping children to develop strategies for self-regulation (Murray and Cooper 1997a; Tsivos et al. 2015). Therefore, the failure of caregivers to respond adequately to their children needs increases fear and confusion (Solomon and George 1999; Carpenter and Stacks 2009) and can induce prolonged levels of cortisol on children (Tsivos et al. 2015). Prolonged levels of cortisol in early infancy modify the HPA axis involved in how infants cope with stress later in live inducing alterations in the PFC and the limbic system (Tsivos et al. 2015), where many glucocorticoids receptors are located.

Moreover, the limbic system is involved in the regulation of emotion and behaviour, attention and inhibitory control, fear and stress reactions, memory, and learning (Steimer 2002; Carpenter and Stacks 2009). Consequently, chronic elevated levels of these hormones can induce negative consequences on emotional regulation, cognitive skills, and brain development (Lupien et al. 2009; Carpenter and Stacks 2009). In addition, infants and young children suffering chronic stress or traumas may have higher levels of cortisol, adrenaline and NA (Carpenter and Stacks 2009) thus engaging in equal consequences. Furthermore, social environment is also important in the context that abnormalities in play behaviour may influence PFC development and later adult behaviour (i.e. children who are hospitalized for extended periods of time (Kolb et al. 2014)).

Various clinical studies have stated that activation of inflammatory response seems to be a hallmark of early life stress since childhood abuse has been related to increased C-protein reactivity in peripheral blood (Danese et al. 2007; Miller et al. 2009) and, as stated above, this inflammatory response produces the release of cytokines in the CNS that is associated with microglia activation (Miller et al. 2009). Furthermore, in adolescents with histories of childhood difficulties increases in IL-6 production previous to onset of depression have been revealed (Miller and Cole 2012; Felger and Lotrich 2013), showing a relationship between chronic stress during childhood, inflammation and depression.

Zeanah and colleagues (Zeanah et al. 2003) carried out a study to assess the implication of early life experiences during childhood by evaluating children raised in institutional settings. Therefore, 3 groups were involved in the study: an institutionalized group involving children who had lived all their life in an institutional setting in Romania; a foster care group, children who were institutionalized at birth and then placed in foster care;

and a never institutionalized group. Children raised in institutional settings in Romania had a lack of experiences that stimulate healthy growth inducing impairments in brain development. These children displayed patterns of physical and cognitive growth that were delayed, and they had different brain activity as well as emotional deficits when compared to children who had never been institutionalized (Korosi 2009). Moreover, the effect of timing of experience was also important in preventing and decreasing these effects; therefore, children who were placed in foster care before they were 2 years old showed patterns of brain activity that were more similar to never-institutionalized children than those placed in foster care after they turned 2 (Marshall et al. 2008). Taken together, these findings suggest that childhood adversity delays brain development and influences the development of psychiatric disorders (O' Mahony et al. 2015).

2.2.2. Adverse early life experiences: Experimental animal studies

In rodents, different models have been assessed to clarify the implications of early life adversity. In this term, maternal separation is commonly used to modify maternal-offspring interactions, and depending on the frequency, the duration, the age at which separation occurs and the quality of maternal care provided to the pups upon being returned to the nest, the consequences will be different (Enthoven et al. 2008; George et al. 2010). Frequently, the protocols of maternal separation last around 2 or 3 weeks after birth and depending on the duration of the maternal separation can be divided in short separation, also called handling (3-15 min), long separation (3-8 h) or deprivation (24 h). Early handling has been suggested as an animal model of resilience to stress and stress-related psychopathology (Levine 1957; Meaney et al. 1988), whereas long maternal separation or maternal deprivation induces detrimental

consequences (van Oers et al. 1997). In addition, a new model called maternal separation with early weaning (MSEW) (George et al. 2010) has been developed in order to reduce any potential compensatory maternal care after maternal separation. In general, maternal separation induces behavioural alterations including increased anxiety (van Oers et al. 1997; Huot et al. 2001; Martini and Valverde 2011), despair-like behaviour (George et al. 2010; Martini and Valverde 2011) and anhedonia-like behaviour (Huot et al. 2001). Cognitive impairments are also induced by this model, including hippocampal-dependant processes (Carlyle et al. 2012; Xue et al. 2013) and emotional memory (Nishi et al. 2013). Moreover, maternal separation produces neurochemical alterations such as an imbalance in the regulation of HPA axis (Millstein and Holmes 2007; Nishi et al. 2013), decreased neurogenesis (Aisa et al. 2009; Lajud et al. 2012) or BDNF levels in the HC (Aisa et al. 2009; Martini and Valverde 2011). Reduced levels of 5-HT1A receptors are also found in maternal separated mice (Leventopoulos et al. 2009; Bravo et al. 2014), as well as higher pro-inflammatory cytokines levels in serum (Réus et al. 2015a) that are associated with increased neuroinflammatory responses (Roque et al. 2015). In order to evaluate institutionalized children experience in animal models, complete maternal deprivation has been performed in rodents. In this model, pups are reared in the absence of maternal contact; therefore pups show social learning impairments, decreased attention, and higher impulsivity (Kundakovic and Champagne 2014). Moreover, pups have decrease neurotrophic factors inducing impaired neural development and reduced plasticity (Kundakovic and Champagne 2014). Similar to the case of attachment in humans, detrimental changes of mother–infant interactions may be a common mechanism in various models of early life adversities (Rice et al. 2008; Brummelte and Galea 2010; Fuentes et al. 2014; Kundakovic and Champagne 2014). These alterations of maternal care can be induced by removal of bedding material from the home-cage

(Rice et al. 2008; Fuentes et al. 2014) or by maternal glucocorticoid administration in order to simulate maternal depression (Brummelte and Galea 2010), both models induce reduced nurturing and increased abusive maternal behaviour (Kundakovic and Champagne 2014). Removing of bedding induces elevated plasma glucocorticoids and increased adrenal weigh. These stressed mice also show dendritic atrophy of pyramidal cells in the HC and a disruption of long-term potentiation in cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) associated with impaired hippocampal-dependent memory (Brunson et al. 2005). In addition, maternal glucocorticoid administration produces not only behavioural alterations including increased despair-like behaviour but also neurobiological changes such as decreased cell proliferation in the HC and increased corticosterone plasma levels (Brummelte and Galea 2010). However, there are other animal models of early life adversity such as early weaning, which induces behavioural and neurochemical consequences in the offspring (Kikusui and Mori 2009; Kikusui et al. 2009) as for example elevated anxiety and aggressiveness and decreased neurogenesis and BDNF level in the HC.

3. Implications of early-life detrimental experiences on the development of depression

Early-life stress, defined as maltreatment or trauma in the form of emotional, physical or sexual abuse; emotional or physical neglect (Chaney et al. 2014), predisposes individuals to develop several psychiatric disorders including depression, bipolar disorder, generalized anxiety disorder, panic disorder, phobias, post-traumatic stress disorder (PTSD) (Kessler 1997; Heim and Nemeroff 2001), and substance use disorder (Dube et al. 2003; Gerra et al. 2009). Indeed, child maltreatment may be perceived as a factor which induces neurodevelopment disruption and, depending in which period occurs can produce serious neurological

alterations that increase the vulnerability to develop certain types of psychopathology mentioned above (Heim et al. 2008; Juruena 2014). Therefore, a large body of neurobiological and neuroendocrine studies has explained that early life stress alters brain development since interfering in critical waves of neurogenesis, synaptic overproduction, and pruning of synapses and receptors (Pechtel and Pizzagalli 2011) induces detrimental regional effects on brain structure associated with psychiatric disorders (Chaney et al. 2014).

Growing evidence shows that stress in the early phases of childhood can cause persistent changes in the ability of the HPA axis to respond to stress in adulthood increasing the vulnerability to develop depression. These alterations seem to be associated with changes in the capacity of glucocorticoids to exert negative feedback on the secretion of HPA hormones through binding to glucocorticoid receptors (Heim et al. 2008; Juruena 2014) and with increased CRH activity that promotes the release of glucocorticoids (Heim et al. 2008). However, recent investigations have proposed that child maltreatment can alter the HPA axis by increasing (Heim et al. 2008; Juruena 2014) or decreasing (Clarke 1993) its activity, depending on age of maltreatment, parental responsiveness, subsequent exposure to stressors, type of maltreatment, and type of psychopathology or behavioural disturbance (Juruena 2014).

In addition, several studies have shown a reduction of hippocampal and PFC volume in depressive patients with a history of childhood maltreatment (van Harmelen et al. 2010; Chaney et al. 2014). These morphometric changes may be due to elevated levels of glucocorticoids which damage both brain regions as they are rich in glucocorticoid receptors (Pechtel and Pizzagalli 2011). Certainly, these alterations are related to cognitive impairments (Gould et al. 2012), consequently deficits

in executive functioning are displayed at adulthood by children who suffered early life stress (Pechtel and Pizzagalli 2011). Other brain areas involved in the mesolimbic system, such as dorsal striatum (Chaney et al. 2014), anterior cingulate cortex (Pechtel and Pizzagalli 2011; Chaney et al. 2014) and amygdala (Kim and Whalen 2009; Pechtel and Pizzagalli 2011) undergo changes produced by early life stress inducing alterations in emotional regulation (Kim and Whalen 2009; Pechtel and Pizzagalli 2011) and reward system (Pechtel and Pizzagalli 2011). Notably, decreased levels of NA were observed in children who were neglected in infancy, as well as sensitization of 5-HT1A receptors (Heim and Nemeroff 2001), suggesting disturbances on monoaminergic neurotransmission.

Furthermore, depressive patients who suffered early life stress display higher inflammatory responses when re-exposed to an acute psychological stress at adulthood (Pace et al. 2006; Lopizzo et al. 2015). In these terms, Miller and Chen (2007) (Miller and Chen 2007) have proposed that when stress takes place while immune system is highly plastic, inflammatory cells including macrophages, microglia and dendritic cells, develop a hypersensitivity that leads to a chronic pro-inflammatory state. This mechanism works as a result of an activation of pro-inflammatory transcription factors such as nuclear factor- kB (NF-kB) and down-regulation of anti-inflammatory transcription factors such as glucocorticoid receptors. However, the process by which early life stress induce the release of pro-inflammatory cytokines is not fully understood (Cattaneo et al. 2015).

Preclinical studies have previously shown that early life stress, can alter brain development by producing changes in neurotransmitters, neuroendocrine hormones and neurotrophins (Kaufman et al. 2000) as well as by modifying synaptic production and pruning (Teicher et al.

2003). Consequently, these experiences induce morphological changes of brain structure similar to those found in humans including HC, anterior cingulate cortex and PFC (Chaney et al. 2014) causing cognitive (Carlyle et al. 2012; Xue et al. 2013) and emotional alterations (Nishi et al. 2013), which are related to impairments on HPA axis regulation (Plotsky and Meaney 1993; Nishi et al. 2013). Therefore, early life stress induces a great impact on subcortical (Andersen 2003) and mesolimbic DA systems, altering their development (Andersen and Teicher 2009). Particularly, exposure to stress enhances DA content and decreases 5-HT turnover in the NAc and causes neural adaptations in the VTA (Andersen and Teicher 2009). Moreover, disturbances in the inflammatory response due to activation of the immune system have been also described in animal models of early life stress (Réus et al. 2015a).

Taking this into account, this thesis project will be focused in the consequences of early life stress on adolescence since it is a period of life with significant psychological and physiological vulnerabilities that increase the risk of developing psychiatric disorders such as anxiety or depression (Dahl 2004; Eiland and Romeo 2013). These characteristics are related not only to the synaptic remodelling of limbic and frontal brain regions (Crews et al. 2007), but also to hormonal alterations (Witt 2007). Furthermore, the late development of the PFC circuits which are associated with executive functions including inhibitory control and sustained attention may guide the tendency of adolescents to impulsivity and to ignore the negative consequences of their behaviour (Casey et al. 2008). Moreover, in order to elucidate the long-term consequences of early life experiences, the implication of these early experiences will be evaluated at adulthood.

Drugs of abuse and substance use disorders

As explained before, negative early life experiences represent a vulnerability factor to develop psychiatric disorders including substance use disorders (Dube et al. 2003; Gerra et al. 2009). Indeed, adolescents suffering mood disorders exhibit two to four times more likelihood to develop drug abuse disorders and addiction compared to adolescents without mood disorders (Riggs 1998). Growing evidence suggests that stress-induced alterations in reward processing may contribute to a higher vulnerability to addictive behaviour (Marinelli and Piazza 2002; Koob 2008). Moreover, depressive patients may take drugs of abuse to alleviate negative affective symptoms (Khantzian EJ 1985; Weiss et al. 2009). Although cocaine is the second illegal consumed drug in our country (WDR-UNODC, 2015), increasing evidence in humans shows that depressive states, which are involved in detrimental early experiences, are likely determinants of cocaine use and abuse vulnerability (Cheetham et al. 2010; Rappeneau et al. 2015). Therefore, this thesis will focus in the influence of early life neglect on the reinforcing and addictive effects of cocaine.

In an initial phase, people take drugs because of its hedonic properties as a result of the activation of the mesocorticolimbic dopaminergic pathway, also known as reward circuit system (Fig. 5). Others contextual factors could influence the positive effects of drug intake for instance the fact of belonging to social groups (peer pressure) with the eventual subsequent transfer of motivation to taking the drug for its reinforcing effects (Koob and Volkow 2010). The reward circuitry system is originated in the VTA which provides dopaminergic innervation to the NAc, one of the most relevant substrates for reward, and also to the amygdala and the PFC (Koob and Volkow 2010) inducing the positive rewarding and reinforcing effects of positive stimulus and directing our behaviour to repeat the

activity inducing reward (Kauer and Malenka 2007; Koob and Volkow 2010). This system is also evolved to promote activities that are essential to the survival for humans as well as other mammals (Sterling 2012). Moreover, natural rewards including food, sex or interpersonal relationships activate this system, although drugs of abuse promote this activation with higher intensity than natural rewards (Wolf 2002). Consequently, the overactivation of the reward system by drugs of abuse induces neuroadaptations that alter the normal function of this system modifying the natural rewarding properties in drug abusers (Koob and Le Moal 2008a). Several evidences suggest the importance of other neurotransmitter in the rewarding effects of drugs such as glutamate, which modulates the reactivity of DA cells and dopamine release in the NAc (Kalivas and Volkow 2005) (Fig. 5).

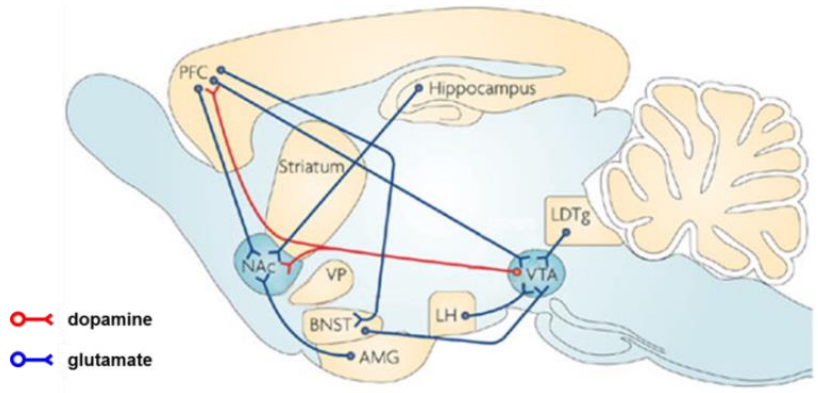


Figure 5. Mesocorticolimbic DA system (modified from Kauer and Malenka, 2007).

Drug addiction can be described as a disorder that progresses form initial motivated behaviour to a compulsive disorder. In this progressive pathological process, the impulsivity reactions are relevant for the development of initial drug use phases and are associated with positive reinforcement (American Psychiatric Association 2013). In contrast, in progressive phases drug taking alleviates a negative emotional state (Koob

2009a) and is related to compulsivity with loss of control in drug consumption (Koob and Volkow 2010). Consequently, the transition from controlled to compulsive drug taking has been associated with a shift in the involvement of striatal subregions (NAc), implicated in the rewarding response to drugs, to the dorsal striatum that is associated with habit formation (Everitt and Robbins 2015). Chronic drug exposure induces behavioural, physiological and neuroplastic changes associated with neuroadaptations in the neurocircuits implicated in the acute reinforcing effects of drugs of abuse, consequently the absence of the drug (withdrawal) induces decreased activity of the mesocorticolimbic DA system and the serotonergic neurotransmission (Koob and Volkow 2010). These modifications during withdrawal cause decreased motivation for non-drug-related stimuli and increased sensitivity to the abuse drug (Melis et al. 2005). In humans, psychostimulant withdrawal is related to fatigue, decreased mood, and psychomotor retardation, whereas in animals is associated with decreased motivation to work for natural rewards and decreased locomotor activity (Pulvirenti and Koob 1993; Barr et al. 1999). In addition, the HPA axis and the brain stress/aversive system mediated by CRF are activated during withdrawal inducing elevated ACTH, corticoids, and amygdala CRF (Koob 2008; Koob and Volkow 2010). Acute withdrawal also produces an aversive or anxiety-like state in which CRF, noradrenergic system and the opioid peptide dynorphin are implicated. Evidence suggests that dynorphin is increased in the NAc in response to dopaminergic activation and that overactivation of the dynorphin system can decrease dopaminergic function, promoting the anxiety state (Koob 2008). The combination of decreases in mesocorticolimbic DA system and increases the brain stress/aversive system provides a negative reinforcement that induces compulsive drug seeking (Wise and Koob 2014).

1. Dopaminergic system in drug abuse

As introduced above, within the systems involved in drug abuse and addiction, this thesis will focus in the dopaminergic system because of its relevance in the drug and natural rewarding properties. In addition, the physiological role of DA in the brain involves, mood behaviour, memory, attention, learning motivation, reward, motor control and endocrine activity, and alterations in this system have been implicated in a number of brain disorders such as mood disorders, substance use disorder, schizophrenia, Parkinson's disease, attention deficit disorder and psychosis (Alcaro et al. 2007).

This system contains mainly two groups of neurons, those medially located in the VTA and those laterally situated in the substantia nigra. The last group mostly projects to the dorsolateral striatum (Graybiel 2000) and is involved principally in motor-related capacities, including locomotor activity and coordination (Robbins and Everitt 1996; Di Chiara 1999). Therefore degeneration of this pathway is associated with Parkinson´s disease (Kravitz et al. 2010). In addition, mesocorticolimbic DA pathway is originated in the VTA a dopamine-rich nucleus located in the ventral midbrain. These dopaminergic axons project and primarily terminate in NAc, in the ventral striatum, but also extend into the amygdala, bed nucleus of stria terminalis, lateral septal area, lateral hypothalamus, and PFC and this system is responsible for motivated behaviour and reward (Volkow and Li 2004; Nestler 2005a). Therefore, as mentioned above, projections from the VTA release DA throughout the circuit in response to a motivationally relevant event such as natural reward or drugs of abuse (Robinson and Berridge 1993). Hence, the release of DA by addictive drugs facilitates learning of drug taking behaviours that play an important role in progressively shaping drug use into drug seeking behaviours that

are difficult to control (Di Chiara 1999; Kelley 2004). However, DA transmission does not fully account for the acute reinforcing effects of all drugs of abuse. Other neurotransmitter/neuromodulator systems including glutamate, γ-aminobutyric acid (GABA), opioid peptides, cannabinoids, and 5-HT are also involved in the rewarding effects of addictive drugs and dopaminergic system modulation (Koob and Le Moal 2008b). In these terms, the PFC sends glutamatergic projections to the NAc whereas the NAc sends GABAergic projections to the ventral pallidum, VTA and substantia migra. Moreover, the ventral pallidum and the VTA send GABAergic efferents to the dorsomedial thalamus which sends glutamatergic projections to the medial PFC (Pierce and Kumaresan 2006). The cholinergic and serotonergic system are also involved in the modulation of the reward system (Kalivas and McFarland 2003). In addition, 5-HT system has been implicated in the acute reinforcing effects of psychostimulant drugs (Nestler 2005b; You et al. 2015). Similarly, the opioid system is involved in the rewarding properties of morphine (Matthes et al. 1996) but also in the addictive effects of most of the drugs of abuse, such as nicotine (Berrendero et al. 2002), cannabinoids (Ghozland et al. 2002), alcohol (Roberts et al. 2000), and cocaine (Becker et al. 2002). Furthermore, several studies have proposed the cannabionoid system as a general modulator of drugs of abuse addiction through a mechanism involving CB1 (Maldonado and Rodríguez de Fonseca 2002; Valverde et al. 2005) and CB2 cannabinoid receptors (Navarrete et al. 2013).

It is important to note that differentiation and anatomical localization of midbrain DA neurons are dependent on the intracellular expression of a wide number of transcription factors, including Nurr1, Pitx3, Lmx1b, and Engrailed 1 and 2 (Lin and Rosenthal 2003; Simeone 2005; Leo et al. 2007; Katunar et al. 2009), although the most extensively studied

transcription factors are Nurr1 and Pitx3 (Leo et al. 2007). Nurr1 (nuclear receptor-related factor 1) is a nuclear receptor of the steroid/thyroid hormone receptor superfamily essential for the differentiation of mid brain dopamine neurons (Jankovic et al. 2005). This transcription factor activates the transcription of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of DA, DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) (Jankovic et al. 2005; Reddy et al. 2011). Nurr1 is expressed under basal physiological conditions in dopaminergic neurons of the VTA and substantia nigra (Xiao et al. 1996; Bäckman et al. 1999) and evidence suggests that Nurr1 expression is regulated by DA signalling, principally through D_2 dopamine receptor (D2R). Consequently, D2R knockout (KO) mice have shown increased Nurr1 expression in midbrain DA neurons (Tseng et al. 2000). However, the role of Nurr1 in the mature midbrain is less clear, although several studies suggest that its expression may be necessary for the correct neurotransmission and maintenance of adult DA neurons (Jankovic et al. 2005; Reddy et al. 2011). The gene encoding Pitx3 (Paired-like homeodomain 3) contains a bicoid-related homedomain and is expressed practically completely in midbrain DA cells (Smidt et al. 1997; Kim et al. 2007). It also activates the transcription genes directly involved in the differentiation of DA neurons (Hwang et al. 2009; Reddy et al. 2011). Nurr1 and Pitx3 seem to control different features of midbrain DA neurons differentiation and survival. In this term, Pitx3 might play an important role in development and/or maintenance of substantia nigra DA neurons, whereas Nurr1 is involved in general midbrain DA neurotransmission (Chung et al. 2005; Simeone 2005).

1.1. Metabolism of dopamine

DA is synthesized in dopaminergic neurons from the precursor amino acid L-tyrosine. The enzyme TH converts L-tyrosine in L-DOPA, which is in turn decarboxylated into DA by the enzyme aromatic L-amino acid decarboxylase (AADC). Dopamine is packed and stored in cytosolic vesicles (VMAT2) and released into the synaptic cleft by fusion of the vesicle with the cellular membrane. In the synaptic cleft DA is eliminated by reuptake via DAT and further catabolized by intracellular MAO to dihydroxyphenylacetic acid (DOPAC), or via extracellular degradation by catechol-O-methyltransferase (COMT) to 3-methoxytryamine (3-MT) which in turn is metabolized to homovanillic acid (HVA) by MAO. Moreover, DA is the precursor of NA by the action of the enzyme dopamine β-hydroxilase (DβH) and phenylethanolamine N-methyltransferase (PNMT) converts NA in adrenaline (Meiser et al. 2013) (Fig. 6).

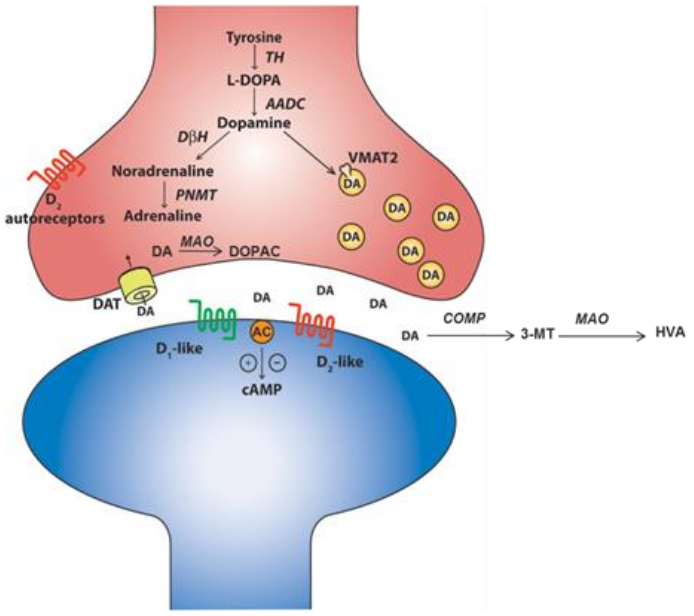


Figure 6. Metabolism of dopamine (Adapted from Sharples et al. 2014).

1.2. Receptors of dopamine

DA receptors are G-protein coupled and mediate slow synaptic transmission through intracellular changes of second messengers. These actions are mediated by the interaction of neurotransmitter with five different receptor subtypes. The DA receptor subtypes are divided into two subclasses: the D_1-type and D_2-type receptors. The D_1-type receptors, D1 and D5, are primarily couple to $G\alpha_s$ and induce the production of cyclic adenosine monophosphate (cAMP). The D_2-type receptors (D2, D3 and D4) are $G\alpha_{i/o}$ couple and inhibit the production of cAMP (Jaber et al. 1996). In the CNS, the various receptor subtypes display specific anatomical distributions, with D_1-like receptors being mainly post-synaptic, whereas D_2-like receptors are both pre- and post-synaptic (Jaber et al. 1996). Moreover, the D_2-like receptors in the presynaptic act as autoreceptors, and are important for negative feedback control of dopaminergic activity (Stoof and Kebabian 1984; Cameron and Williams 1993). In addition, D_1 dopamine receptor (D1R) and D2R are the most abundant subtypes in the CNS, and appear to be expressed in brain regions which receive dopaminergic inputs (Jaber et al. 1996). Both are large placed in dorsal striatum, olfactory tubercle and NAc, and in lower areas such as HC, neocortex, hypothalamus and thalamus (Civelli et al. 1993; Missale et al. 1998).

1.3. Drug-induced adaptive changes in the dopaminergic system

Several studies have demonstrated that chronic exposure to different types of drugs induces adaptive changes in different components of dopaminergic system.

One of the most relevant changes is the down regulation of D2R in discrete brain areas (Thanos et al. 2001; Nader et al. 2006). In rodents, low levels of D2R in striatum are related to impulsivity and predict escalating and compulsive administration of cocaine (Everitt et al. 2008). In addition, human studies have shown reductions in D2R availability in ventral and dorsal striatum for most of the drugs in addict patients (Volkow and Baler 2014). Indeed, individuals with reduced D2R levels in the striatum seem to present a high vulnerability to develop drug addiction (Volkow et al. 2002). Moreover, this reduction in drug abusers has been associated with decreased activity in the PFC, including anterior cingulate and orbitofrontal cortical regions. The anterior cingulate and orbitofrontal cortex are necessary for self-control and for processing salience attribution, and their disruption is associated with a propensity for impulsive and compulsive behaviours (Volkow and Fowler 2000). In addition to down regulation of D2R, cocaine-addictive individuals have a decrease in DA release inducing a lower dopaminergic activation for natural reward, therefore cocaine may help them compensate this deficits, increasing the vulnerability to consume (Volkow et al. 1999).

Regarding D1R, the consequences of repeated drug exposure on D1R have not been consistent. Several studies have exposed that chronic cocaine enhanced D1R signaling (Pascoli et al. 2012), whereas others experiments have shown decreased D1R excitability (Kim et al. 2011). These discrepancies may be related to the experimental conditions, since each study evaluated the D1R signaling at different timings after cocaine administration.

Interestingly, previous studies using animal models and human cocaine dependent users have reported that DAT expression activity is increased, decreased, or showed no changes after repeat cocaine administration (Cass et al. 1993; Izenwasser 2004).

Finally, chronic cocaine produces long-term adaptations in different transcriptional factors associated to dopaminergic signaling. Therefore, cocaine abusers display decreased Nurr1 expression in DA neurons. Nurr1 plays a role in regulating the transcription of DAT and DAT gene expression is decreased in the DA neurons of cocaine abusers (Bannon et al. 2002), suggesting that Nurr1 is involved in controlling human DAT gene expression and adaptation to repeated exposure to cocaine (Jankovic et al. 2005). Similarly, rats chronically treated with cocaine showed a down-regulation of the expression of Nurr1 mRNA and protein in the ventral midbrain, as well as in Pitx3 transcription and protein levels (Leo et al. 2007).

2. Cocaine as a drug of abuse: history of its recreational use, epidemiology, and mechanism of action

2.1. Brief history of recreational use of cocaine

Cocaine is a natural substance obtained from the coca plant, Erithroxylon Coca, and natives from South America have used coca plant for centuries to allay fatigue, sustain performance, and treat a large variety of diseases by chewing and sucking the leaves (Haddad 1978). Coca was introduced in Europe by the explorers and then by the botanists (Mortimer 1901; Gerstein and Harwood 1992). In 1855, Albert Niemann isolated the main alkaloid of the plant and called it “cocaine”. The availability of the purified form of the drug cocaine led to research on its effects, and it was also recommended for a range of illnesses (Stolberg 2011). During the late 19th century, several medical doctors started to consume cocaine and to prescribe it to their patients, and realized of its harmful effects; Sigmund Freud wrote an essay about cocaine´s effects (Freud 1984). However, several pharmaceutical companies started to sell different preparations

with cocaine that people could buy in chemists without medical prescription, as well as beverages including Coca-cola. In 1909, Coca-cola Company eliminated cocaine of its preparation and substituted it by caffeine (Stolberg 2011). Finally, in North America, Cocaine was classified as an illicit drug in 1914 (Harrison Narcotics Tax Act, 1974) whereas in Spain, cocaine was considered illegal in 1944 (Herrero Álvarez S 2001).

Nowadays, cocaine is the second most consumed illicit drug after cannabis (WDR-UNODC, 2015).

2.2. Epidemiology

Cocaine is used by around 17 million people worldwide meaning nearby 0.4 percent of the global population between 15 to 64 years old (WDR-UNODC, 2015). In the European Union around 1.9% among the 15-34 years old population has recognized to have consumed cocaine in the last year. Indeed, the United kingdom (4.2 %) and Spain (3.3%) are the countries with highest cocaine consumption prevalence (Mounteney et al. 2015), fortunately the prevalence around the world has declined slightly over the past decade (WDR-UNODC, 2015**).**

In Spain, the medium age of beginning cocaine´s chronic use is around 21 years old, and the prevalence in men is 3.5 times higher than in women (Ministry of Health, Press release, 2015). Moreover, cocaine is the illegal drug most often associated with visits to hospital emergency departments in Spain. Therefore, in 2009 the 51.1% of the illicit drug-related cases in emergency departments at hospital had a direct relationship to cocaine (Mena et al. 2013). In addition, cocaine is the illicit drug responsible for most drug-deaths in Spain (González Llona et al. 2015).

Cocaine is the second illicit drug most consumed after cannabis (WDR-UNODC, 2015). Furthermore, alcohol, which is the most consumed drug during adolescence (WHO, 2014), is frequently consumed together with other drugs of abuse, particularly psychostimulants like cocaine (Winstock et al. 2001; Barrett et al. 2006).

As stated above, Spain is one of countries in which more cocaine is consumed causing serious health pubic problems (Mena et al. 2013; González Llona et al. 2015). Therefore, cocaine studies are one of our research priorities in our group.

2.3. Mechanism of action

Cocaine directly increases the mesocorticolimbic dopaminergic signal by blocking the presynaptic DAT (Ritz et al. 1987) (Fig. 7). In particular, dopaminergic projections from the VTA to the NAc are the most strongly identified with drug-related reinforcement, although other brain areas are also involved such as amygdala and PFC (Volkow et al. 1999; Koob and Volkow 2010; Dong and Nestler 2014). Functional neuroimaging studies have further long-established the importance of the mesolimbic pathway and DA release to the cocaine-induced reward (Koob and Volkow 2010). In addition, cocaine also inhibits the reuptake of 5-HT and NA by binding to their transporters, although the level of inhibition is lower than that of the DA system (Ritz et al. 1987).

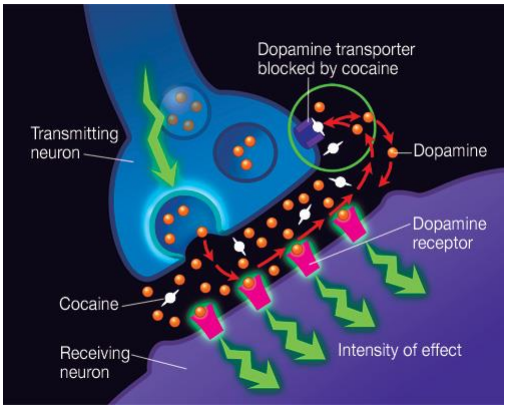


Figure 7. **Cocaine blocks the presynaptic dopamine transporter (DAT)** (Image from National Institute of Drug Abuse of United Stateds of America).

2.4. Pharmacological effects

Cocaine pharmacological effects depend on its mechanism of action as well as the dose and route of administration. Hence, intravenous or smoked it produces marked intense pleasurable sensation characterized as a 'rush', that is thought to be a powerful motivation for the abuse of these drugs, whereas intranasal administration of cocaine also produces euphoric and stimulant effects that last approximately 30 min (Belin et al. 2009). Luo and colleagues have been proposed that stimulation of D1R and D2R only occurs when drugs achieve fast peak concentrations, whereas as the concentration of DA starts to decrease, D2R are predominantly stimulated (Luo et al. 2011). This may also explain why routes of administration that achieve faster and higher drug levels in the brain, such as mentioned above, are more rewarding and addictive than routes of administration that result in slow brain uptake, like oral administration (Volkow and Morales 2015). In addition, cocaine has high abuse potential and can induce addiction, although only 15-20% of users become addicted to this drug, clinical observations show that controlled use often shifts to more compulsive use (Anthony et al., 1994), mainly

when the access to the drug is easier or when the route of administration is faster (Everitt et al. 2008).

Long-term cocaine consumption triggers various forms of synaptic plasticity that can result in strengthening or weakening of synaptic connectivity in various brain reward regions (Grueter et al. 2012). Cocaine-induced neuroplasticity involves the same molecular mechanisms implicated in long-term potentiation (LTP) and long-term depression (LTD) that underlie learning and memory (De Roo et al. 2008) affecting particular neurotransmitter system including monoamines and glutamatergic signalling. These synaptic modifications induce a long-lasting molecular memory for the drug's rewarding and conditioning effects that will modify subsequent behaviours (Hyman et al. 2006). In these terms, DA regulates excitatory synaptic plasticity both by increasing and decreasing synaptic strength through LTP and LTD, respectively. Synaptic strength is controlled by the insertion or removal of glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or NMDA and by changes in the subunit composition of AMPA receptors. Particularly, the insertion of high-calcium permeable AMPA receptor (GluR2 subunit) increases AMPA receptor conductance (Liu and Cull-Candy 2000; Guire et al. 2008) and contributes to the drug-induced increases in AMPA/NMDA ratio associated with LTP in models of addiction (Conrad et al. 2008). Therefore, several experimental studies have demonstrated that in the VTA after one dose of cocaine some AMPA receptor subunits are exchanged for high-calcium permeable subunits (GluR2) increasing the ratio AMPA/NMDA. After a week of a passive injection or months after self-administration, the baseline composition is restored (Ungless et al. 2001; Lüscher and Malenka 2011) showing the differences between non-contingent and contingent drug-administration. Furthermore, cocaine also causes neuroplastic adaptations in the NAc,

inducing a reduction in the AMPA/NMDA. These alterations are implicated in habit learning and in the automatic cocaine consumption triggered by repeated cocaine exposures (Everitt et al. 2008; Lavaur et al. 2009). PFC is also modified by cocaine-induced neuroplasticity and is associated with cue-induced drug craving and drug-seeking behaviour (Nestler 2005b; Lüscher and Malenka 2011). Moreover, chronic cocaine self-administration can induce long-lasting neuroplastic modifications that are maintained during months (Chen et al. 2008).

Furthermore, chronic use of cocaine produces tolerance to its rewarding effects and results in the need to consume higher doses to produce the same feelings of euphoria contributing to develop addictive behavior facilitating to the spiralling distress/addiction cycle propose by Koob and Le Moal (Koob and Le Moal 2001). Therefore, as cocaine use and duration increases, the positive reinforcing effects are decreased resulting in dysphoria states (Fischman et al. 1985). Presumably, the DA increases triggered by cocaine activate D2R auto-receptors inhibiting DA cell firing and DA release (Bello et al. 2011), that may explain why the intensity of the cocaine ‘‘high’’ is reduced with subsequent administrations, whereas the motivation to continue to take the drug persists (Volkow and Morales 2015).

Consequently, although cocaine does not induce physical dependence, when the drug is prevented after chronic use, results in a deregulation of dopaminergic systems that contributes to anhedonia, increased impulsivity, and relapse after withdrawal (Robinson and Berridge 1993). Therefore, cocaine withdrawal modulates DA neuronal activity in the VTA by modifying the burst firing pattern inducing a hypodopaminergic state (Hatzigiakoumis et al. 2011). In fact, early stages of cocaine withdrawal may reduce bursting activity of VTA DA cells and these

alterations may be associated with a decrease in the sensitivity of D2R induced by chronic cocaine exposure (Shi et al. 2008; Hatzigiakoumis et al. 2011). Chronic cocaine dependence also induces a distress state marked by HPA axis deregulation and enhanced sensitivity to negative emotion (Sinha et al. 2003; Fox et al. 2009). CRF, noradrenergic system and the opioid peptide dynorphin are also implicated in the dysphoric state induce by chronic cocaine use and manifested during withdrawal (Wise and Koob 2014). Moreover, individuals chronically exposed to cocaine present long-lasting memory deficits that are characterized by poor immediate and retardant verbal recall and recognition and also a selective reduction in working memory (Fox et al. 2009). Animal studies demonstrated that these deficits were not only related to increased levels of glucocorticoids, but also to the effects of cocaine on cell proliferation and neurogenesis in adult rat hippocampal DG (Sudai et al. 2011).

In addition, several animal models have been developed in order to elucidate the neurobiology of addiction, as well as the rewarding and reinforcement properties of drugs of abuse. Therefore, much of the recent advance in understanding the neurobiology of addiction has derived from the study of animal models of addiction, although no animal model of addiction completely emulates the human disorder (Koob and Volkow 2010). The most validated models are the conditioned place preference (CPP) that allows to evaluate the rewarding properties of drugs of abuse (Valverde et al. 1996; Roux et al. 2001; Prus et al. 2009), the locomotor sensitization which evaluates the capacity of increasing the locomotor activity after repeated administration of the drugs of abuse (Robinson and Berridge 1993; Robinson and Berridge 2001) and the self-administration procedure which evaluates the reinforcement properties of the drugs of abuse (Thomsen and Caine 2005). These models will be detailed in section 7 of Methodology Section.

3. Cocaine abuse and depressive disorders

Evidences from epidemiological, clinical and preclinical studies have shown a robust association between emotional disorders and drug addiction (Cheetham et al. 2010). Individuals with emotional disorders including depression or PTSD have higher vulnerability to develop drug abuse (Merikangas et al. 1999). Similarly, emotional disorders have been observed in those individuals suffering drug abuse (Volkow 2004; Polter and Kauer 2014). Results from representative population survey of United States of America have indicated that 30–50 % of individuals with psychotic illnesses (Regier et al. 1990; Kendler et al. 1996) or emotional disorders (Grant et al. 2005; Hasin et al. 2005) have a comorbid alcohol use disorder or non-alcohol drug use disorder. Moreover, a recent study has shown that 10.9 % of depressive illness patients were also registered with a comorbid substance use disorder (Nesvåg et al. 2015).

This comorbidity is accompanied by greater functional disability, longer illness duration, less social competence, and higher service utilization (Lalanne et al. 2014). In addition, the fundamental mechanisms of comorbidity between emotional disorders and substance use disorder are not fully understood (Volkow 2004). One hypothesis to explain the high prevalence of the co-occurrence of these two disorders may be the overlapping environmental, genetic and neurobiological factor (Volkow 2004). Moreover, drugs of abuse are used in an effort to self-medicate during depression in order to relieve the feelings of sadness and anhedonia or to alleviate the side-effects of antidepressants (Khantzian EJ 1985; Weiss et al. 2009). However, the exposure to chronic drugs of abuse might induce neurobiological changes that increase the risk of depression (Volkow 2004).

Comorbidity between depression and drugs of abuse is well established for psychostimulant drugs, such as cocaine. Therefore, depressive patients show considerably higher rates of lifetime cocaine use, whereas cocaine abusers display greater lifetime prevalence of depression (Conway et al. 2006; Rappeneau et al. 2015).

The risk to development an emotional or anxiety disorder after stress is positively associated with the risk for substance use disorders (Sinha 2008) suggesting that there are common substrates for vulnerability to addictive and affective disorders (Polter and Kauer 2014). Acute stress is related to episodes of depression and to relapse in drug abuse, whereas chronic stress is a common element in the environmental variables of depression and drugs of abuse (Schneiderman et al. 2005; Kanter et al. 2008; Sinha 2008). In fact, clinical and experimental studies have shown that both acute and chronic stressors affect the dopaminergic system and reward mechanisms and can induce anhedonia (Cabib and Puglisi-Allegra 2012; Pizzagalli 2014). Moreover, the dopaminergic system mediates the reward and reinforcing effects of drugs of abuse (Koob and Volkow 2010). Recently, the kappa opioid receptor has been proposed as a modulator of the DA system associated with mood regulation and reward and reinforcement properties of the drugs of abuse (For review see Lalanne et al. 2014).

In humans, there is considerable evidence from prospective and longitudinal studies to support the effects of stress during childhood or adolescence on drug use initiation, escalation and addiction (Sullivan et al. 2006; Somaini et al. 2011). Furthermore, beginning substance use during adolescence reduces the transition from drug use to addiction (Clark et al. 1998). Therefore, negative childhood events such as loss of a parent, physical violence and abuse, neglect or isolation and social status have

been related to an elevated risk of exposure to drugs during adolescence (Dube et al. 2003; Gerra et al. 2009). Considering this evidence, Teicher and co-workers found that young adults who had been maltreated as children displayed alterations in the connectivity of different cortical regions that may increase their risk for substance use. The differences could compromise basic social perceptual skills, ability to maintain a healthy balance between introversion and extroversion, and ability to self-regulate their emotions and behavior (Teicher et al. 2014).

In addition, neurobiological alterations induced by chronic exposure to stress during childhood, involving different neurotransmitters function and HPA axis function may increase substance use susceptibility later during the adolescence in humans (Oswald et al. 2005; Duval et al. 2006). Enhanced DA release in the NAc has been stated in early stress exposure (Kalivas and Duffy 1995), chronic stress and increased levels of glucocorticoids. Nevertheless, chronic exposure to glucocorticoids inhibits both DA synthesis and turnover, hence suggesting that deregulation of the HPA axis can alter dopaminergic function (Pacak et al. 2002). Furthermore, several studies show that the alterations of HPA axis function and stress system play an important role in the evolution from casual use of substances to the incapacity to stop the chronic use (Sinha 2008). Mainly, these neurobiological alterations have been related to childhood history of adverse experiences and low perception of parental care among both cocaine and heroin users (Sinha 2008).

Taking this into account, evidence from animal studies supports the notion that acute or chronic exposure to stress increases initiation and escalation of drug abuse (Sinha 2008). Therefore, models such as social defeat, social isolation, electrical foot-shock, and maternal separation enhance self-administration of cocaine as well as its rewarding properties and

locomotor sensitization at adulthood (Burke and Miczek 2014). Only few studies have evaluate the effects of stress in rodents on addictive behaviour during adolescence (Doremus-Fitzwater and Spear 2010; Rodríguez-Arias et al. 2015), therefore further investigation is needed in this area of research. However, it is worth mentioning that the behavioural and neurobiological consequences of chronic stress models depend on the frequency, duration and lifespan period in which stress takes place and therefore so will the consequences obtained by different approximations.

OBJETIVES

Considering the theoretical frame that we have exposed in the Introduction Section, the **overall objective** of this thesis was to evaluate the short and long-term consequences of a validated model of early life neglect and social enrichment respectively on emotional alterations and drug abuse during adolescence and adulthood. Behavioural studies and neurochemical parameters were evaluated for achieving our main objectives

The **specific objectives** were the following:

A) To evaluate the short and long-term consequences of validated models of early life neglect and social enrichment on emotional alterations and neurochemical changes related to them.

1) To establish the validated models of early life neglect, maternal separation with early weaning (George et al., 2010), and social enrichment, communal nest (Branchi et al., 2006a), in our experimental conditions.
2) To evaluate the spontaneous maternal behaviour along the first 16 days after pups birth.
3) To determine behavioural consequences in the offspring at adolescence and adulthood. Specifically spontaneous locomotor activity, anxiety-like, despair-like and anhedonia-like behaviour.
4) To evaluate cognitive alterations, in particular emotional learning and memory impairment.
5) To study the inflammatory response at peripheral and central levels in order to establish a link between emotional alterations and the immune system. Determination of IL-6 serum levels and evaluation of microglia activation and astrocytes in PFC and HC, brain areas related to emotional control.

6) To evaluate the tryptophan-kynurenine pathway in the PFC and the HC to study the relationship between depressive state and the imbalance of this metabolic route.

B) To determine the impact of these early life experiences on the effects of cocaine.

1) To evaluate the rewarding properties of cocaine using the conditioned place preference. To study the plasticity of dopaminergic reward system in our experimental conditions.
2) To examine the effects of these early life conditions on cocaine-induce locomotor activity (acute effect) and cocaine-induced sensitization (repeated treatment).
3) To study the reinforcing effects of cocaine using the operant self-administration model.

METHODOLOGY

1. Animals

We used 70 male and 70 female outbred CD1 mice as breeders for this study (purchased in Charles River, Barcelona, Spain), and shipped to our animal facility, UBIOMEX, PRBB. Animals were 10 weeks old at the start of breeding and were housed individually in standard cages in a temperature- (21°± 1°C), humidity- (55% ± 10%), and light-cycle-controlled room. The room was lit between 8:00 h and 20:00 h, and experiments were conducted during the light phase (8:30 h to 15:00 h), except for the evaluation of maternal behaviour, as indicated, and for the self-administration procedure, in which the room was lit between 20:00 h and 8:00 h. Food and water were available *ad libitum* except during behavioural testing of the offspring. Mice were allowed to acclimatize to the new environmental conditions for at least one week before starting the experiments. Every effort was made to minimize animal suffering and reduce the number of animals used. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research, and were approved by the local ethics committee (CEEA-PRBB).

2. Rearing conditions

Mice were randomly assigned to one of the three different experimental groups, standard nest (SN), MSEW and CN. For SN and MSEW, breeding pairs (one male, and one female) were housed in Plexiglas cages (36.9 x 15.6 x 13.2 cm). For CN group (Branchi and Alleva 2006), 1 male and 3 females were used to form breeding groups and housed in a 42.5 x 26.6 x 18.5 (cm) Plexiglas box. In all the cases, the males were removed when the females were about 10 days pregnant. Pregnant females were observed daily at 9 and 17 h for parturition. For each litter, the date of birth was

designed postnatal day (PD) 0. In the MSEW group, offspring were separated from their mothers for 4 h per day on PD2-5 (9:30-13:30 h) and 8 h per day on PD6-16 (9:30-17:30 h). For separation, mothers were moved to another cage, while the offspring remained in their home cages with a heating blanket (32-34ºC) for thermoregulation. After removing the mothers, offspring were taken to another room to avoid their mothers to become stressed from hearing their vocalizations (George et al. 2010). Pups were weaned at PD17, and to facilitate their access to food and avoid a possible dehydration, wet regular chow and hydrodrogel (Bio-Services, Uden, The Netherlands) were provided in their homecages until PD21. The CN group consisted in three females giving birth and rearing the same litter (Branchi and Alleva 2006) and pups were weaned on PD25. In each female trio, there was a discrepancy of up 2-4 days in the age of the pups. In the SN group, offspring remained with their mothers for 21 days and were then weaned (PD21). Cages remained untouched until PD10, when they were cleaned. After weaning, offspring were housed in groups of 4 to 5 animals of the same sex. For the behavioural and neurochemical experiments at adolescence, 5, 4 and 6 females were assigned to the MSEW, SN and CN groups, respectively. We observed no significant differences between groups in the total number of offspring, or the number of males or females. Average litter size was 13 (53% male). A different group of mice was used to evaluate adult behavioural parameters. In this case, 5 females were assigned to MSEW and SN group and 6 females to CN group, and we observed no differences between groups in the number or sex of the offspring (average litter size, 12; 56% males). For reward and addictive behaviour studies, 16 females were assigned to MSEW and SN group, but also not differences were found in the number or sex of the offspring (average litter size, 14; 57% males).

3. Maternal care

As explained in the Introduction Section, the maternal care display by mothers can modulate the brain development including emotional parameters and cognitive skills (Lupien et al. 2009; Korosi 2009). Therefore, we recorded the biological mother's spontaneous maternal behaviour 3 times per day (8:15 h, 17:30 h and 20:15 h) from PD1 to PD16 according to an adapted version of a previously described protocol (Dimitsantos et al. 2007; Fodor et al. 2012). The long break between the morning and afternoon maternal care evaluation session was consistent with the 8 h maternal separation period performed during PD6-16 (9:30-17:30 h). Observations of maternal care behaviour were performed at three periods of the day, at 8.15 h, 17.30 h and 20.15 h and were recorded on-line by an observer who remained silent in the room. Within each observation period, the behaviour of each mother was scored 25 times spaced 3 min each one (25 observations x 3 periods per day x 16 days = 1200 observations/mother). The following behaviours were scored as present or absent and quantified in a check list: 1) mother licking and grooming any offspring (body + anogenital region); 2) mother nursing offspring in an arched-back posture with rigid limbs ("high kyphosis"); 3) mother nursing in a "blanket" posture, i.e. lying on the offspring or her limbs are rigid but she has a low dorsal arch posture ("low/partial kyphosis"); 4) mother nursing in a "passive" posture ("supine nursing"), i.e. lying on her back or side while the offspring nurse; and 5) mother "off" offspring (no maternal contact).

4. Behavioural procedures

The offspring's body weight was recorded at PD10, 17, 30, 62 and 83 to evaluate their nutritional status. The mice were observed to evaluate spontaneous behaviour during adolescence (starting at PD30) with a

maximum discrepancy of 4 days due to date of birth. For some experiments, the behaviour of adult mice (starting at PD90) was also evaluated (spontaneous locomotor activity, elevated plus maze (EPM), tail suspension test (TST), saccharin test and passive avoidance test) in order to investigate the long-lasting consequences of these rearing conditions. All tests were carried out between 8:30 and 15:00 h. Animals were transferred to the experimental room ≥30 min before the test to acclimatize them to the test environment (Fig. 8). Distinct groups of mice were used to evaluate adolescent and adult behaviour for all the experiments performed. The spontaneous locomotor activity, the EPM and the TST were performed in the same group of mice. Distinct groups of animals were used for the experiments related to pain threshold, passive avoidance model and the saccharin test, respectively.

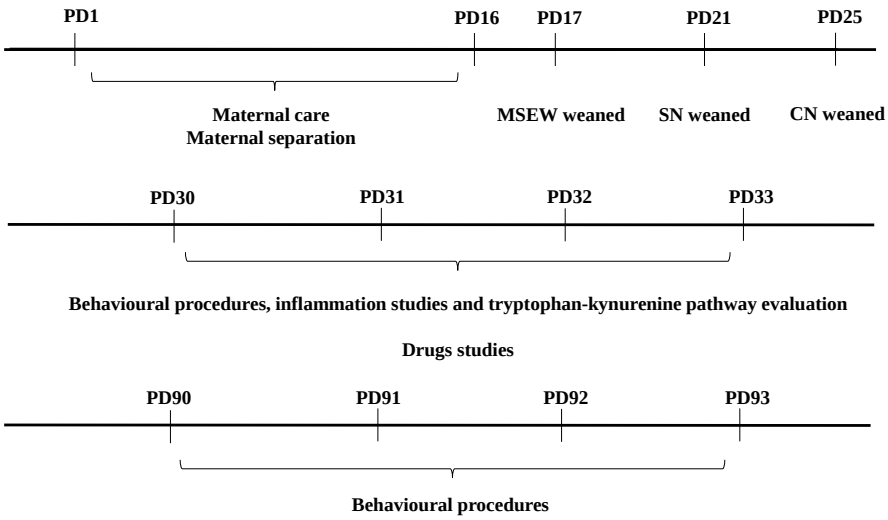


Figure 8. Experimental schedule. Postnatal day (PD); maternal separation with early weaning (MSEW); communal nest (CN); standard nest (SN).

4.1. Spontaneous locomotor activity

The spontaneous locomotor activity of mice is a parameter primarily controlled by the brain and influenced by stress (Thomas et al. 2011). Therefore, spontaneous locomotor activity can be an index of psychomotor retardation or apathy, both symptoms of depressive-like behaviour (Sobin and Sackeim 1997; Lavretsky et al. 1999), but also an indirect sign of anxiety (Strekalova et al. 2005). Animals were evaluated for spontaneous activity, as previously described with minor modifications (Ros-Simó et al. 2012) for 20 min on PD31-33 and PD91-93 respectively using locomotor activity boxes (24 x 24 x 24 cm) (LE8811 IR, Panlab S.L., Barcelona, Spain) (Fig. 9) in a low luminosity room (20 lux) with white noise. To detect the mouse movement, the boxes were equipped with photocells beams; each photocell interruption was counted and registered by a computer. Horizontal (deambulations) and vertical (rearings) activities were measured as the total number of beam breaks in the lower or upper photocell layer respectively.

Figure 9. Locomotor activity box

4.2. Elevated plus maze

In the paradigms used to evaluate anxiety, mice are exposed to a conflict between the natural impulse to explore a novel environment and the innate behaviour to avoid aversive environments such as lit compartments, wide places or elevated mazes (Belzung and Griebel 2001). The EPM is based

on the innate aversion of rodents to height. This maze consisted of a black plastic apparatus with four arms (29.5 x 6 cm) set in a cross from a neutral central square (5 x 5 cm). Two opposite arms had vertical walls (closed arms); and the two other opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and illuminated from the top (100 lx) (Fig. 10). Each mouse was placed in the center of the maze for a 5 min period. Percentage of entries in the open arms and percentage of time spent in open arms were measured as an index of anxiolytic behaviour. As a measure of general activity, total entries were also quantified. An entry was recorded when the animal placed four paws into the arm. At PD32-35 and PD92-95 anxiety-like behaviour was measured in the EPM (Panlab, S.L.,Barcelona, Spain) using a similar procedure to that reported previously (Pellow et al. 1985; Simonin 1998).

Figure 10. Elevated plus maze

4.3. Tail suspension test

TST is a model to evaluate despair behaviour based on the evidence that when a mouse exposed to a stressful situation from which it cannot escape (mice is suspended by its tail), the initial reaction consists in trying to escape, followed by an immobile posture related to despair-like behaviour (Fig. 11). Moreover, the immobile posture is reversed by administration of antidepressants (Steru et al. 1985; Powell et al. 2011). Each mouse was suspended individually (using adhesive tape attached 1 cm from the tip of the tail) 50 cm above a bench top for 6 minutes. The time in which the

animal was immobile during this interval was recorded as an index of despair behaviour. Mice underwent the TST on PD33-36 and PD93-96, as described previously (Steru et al. 1985; Aso et al. 2008).

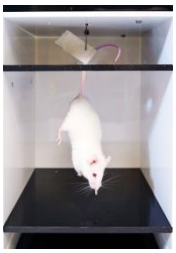


Figure 11. Tail suspension test

4.4. Saccharin test

Saccharin test is a behavioural model based on the mice preference for drinking sweet solutions. Therefore, when mice have access to a solution of saccharin, the normal behaviour is to prefer drinking saccharin over water due to the natural preference of mice for sweet solutions (Berridge 2007). This test is currently used to evaluate the anhedonia-like behaviour, a core symptom of depression (D'Aquila et al. 1997; Harkin et al. 2002; Strekalova et al. 2004). Mice subjected to stress show a decrease in the consumption of saccharin solution related to a decrease in sensitivity to reward (Willner et al. 1987). These effects can be reversed by the treatment with antidepressants (Powell et al. 2011). Saccharin test was performed on PD34-40 and PD94-100 in a different group of mice. Mice were individually housed one week before starting the experiments and exposed to a saccharin solution (0.33% w/v) (Sigma-Aldrich, Madrid, Spain) and tap water during 72 h (Fig. 12), according to (Lu et al. 2005b) and (Disse et al. 2010) with minor modifications. The position of the bottles was switched each 24 h, in order to prevent a possible effects of side preference in drinking behaviour. No previous food or water

deprivation was applied to the mice before the test. The consumption of water and saccharin solution was evaluated simultaneously to all experimental groups by weighing the bottles every 24 h. Mice were also weighed every day. A control cage without animals was placed to control the amount of liquid spontaneously loss from the bottles. The saccharin preferences were calculated at the time points 24 h, 48 h and 72 h after the exposure to saccharin solutions, according to the following ratio: saccharin intake (g)/[saccharin intake (g) + water intake (g)] × 100%. Additionally, the number of grams of saccharin consumed per kilogram of body weight was also calculated for the three time points above mentioned.

Figure 12. Saccharin test

4.5. Electrical nociceptive threshold

A separate group of adolescent mice (PD30) were used to assess the pain threshold to electrical stimulus in accordance to a previously procedure (Martin et al. 2002; Tsuji et al. 2003) with minor modifications. We used to perform this procedure the dark compartment of the passive avoidance device (see *4.6. Passive avoidance section*). Mice were allowed 5 min to habituate to the compartment before a range of inescapable shocks exposure. During this period, locomotor activity was evaluated by measuring the number of rearing and the squared crossed (4.6-4.8 cm). The electric foot shock delivery consisted in 10 shocks spaced 30 s at 0.5

mA and 3 s of duration. The number of jumps (all paws off the grid floor) and vocalisations were recorded. The mice used to evaluate the electrical nociceptive threshold were not used for other behavioural procedure. This test was only developed in MSEW and SN group in order to ensure that the differences found in the passive avoidance test were due to emotional memory impairments and not to a different electric pain threshold in the mice exposed to different rearing conditions. In this case, CN group was not evaluated since no changes regarding SN group were observed during the passive avoidance test in adolescent mice.

4.6. Passive avoidance test

The passive avoidance test is used to evaluate learning and memory based on the natural preference of mice for a dark environment, and the association between an aversive stimulus, in this case an electric foot shock, and the preferred environmental context (Saavedra et al. 2013). This test is related to emotional memory (LeDoux 1993), being the central nucleus of the amygdala one of the main brain areas involved in this task (LeDoux 1993; Phelps 2004). Two separate groups of animals underwent the passive avoidance test, in adolescence (PD30-33) and adulthood (PD90-93). This test was conducted as previously described (Saavedra et al. 2013) with minor modifications. The mice involved in this test did not undergo any other behavioural procedure. The experiment was conducted in a device divided into a weakly (black walls) and brightly lit (white walls) compartment (2-5 and 160 lux, respectively; dimensions, 19 x 19 x 27 cm) (Panlab S.L., Barcelona, Spain). The dark chamber had a stainless steel grid floor for shock delivery (Fig. 13). This test consists in two trials, the acquisition trial in which the black compartment was paired with the electric foot shock (learning) and the retention trial in which the emotional memory was evaluated. On the acquisition day, each mouse was placed

into the bright compartment. A sliding door between the compartments was opened after 30 s, and the latency to enter the dark compartment was recorded for up to 90 s. Upon entering the dark compartment, the door was closed and mice received a foot shock (0.5 mA, 3 s), and were immediately removed from the apparatus. The mice were returned to the brightly lit compartment 24 h later, and the procedure was repeated but omitting the foot shock (retention trial). The latency to enter the dark compartment was recorded. The retention test ended when mice stepped completely into the dark compartment, or failed to cross within 300 s. Retention latency is an index of memory since mice that learn the task avoid the compartment previously paired with the shock, and show greater latency to enter the dark compartment.

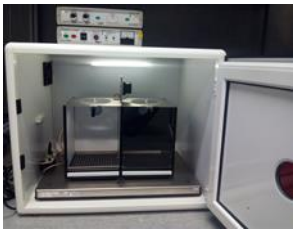


Figure 13. Passive avoidance

5. Studies conducted to evaluate inflammatory reactions in mice exposed to different rearing conditions

5.1. Evaluation of peripheral inflammatory response. Interleukine-6 determination

On PD30, blood samples were collected by decapitation of animals at basal conditions. Samples were centrifuged for 35 min at 1500 g, 4ºC, and serum was retrieved and frozen at –80°C. IL-6 was measured with an enzyme-linked immunoabsorbent assay kit according to manufacturer's instructions (BD Opt EIA Mouse IL-6 ELISA kit, catalogue no. 550950; BD Biosciencies, San Jose, CA).

5.2. Evaluation of neuroinflammatory responses. Microglia and Astrocytes

On PD30, we evaluated microglia activation and astrocytes in naïve animals to study neuroinflammatory responses in the PFC and three hippocampal regions, namely the CA1 and CA3, and the DG. We evaluated the presence of ionized calcium-binding adapter molecule 1 (Iba1) in microglia and glial fibrillary acid protein (GFAP) by immunofluorescence using rabbit polyclonal anti-Iba1 staining (1:300; Wako Pure Chemical Industries, Ltd., Japan) and rabbit polyclonal anti-GFAP (1:500; DakoCytomation, Glostrup, Denmark) respectively, as previously reported (Touriño et al. 2010; Ros-Simó et al. 2012). Mice were anesthetized with a ketamine/xylazine (Merial and Sigma Aldrich, Barcelona, Spain) mixture (100 and 20 mg/kg, respectively), and perfused transcardially with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (Merk KGaA, 64271 Darmstadt, Germany). The brain was removed and postfixed in the same solution for 4 h and cryoprotected in 30% v/v sucrose (Merk KGaA, 64271 Darmstadt, Germany) in 0.1 M

in PB (pH 7.4; 24h at 4°C). After freezing in dry ice, the brain was sliced into 35 μm coronal sections. A mouse brain atlas (Paxinos and Franklin 2004) was used to identify the anatomical location of the PFC, CA1, CA3 and DG (3 samples per area per mouse, evaluated bilaterally). Floating brain sections were washed three times in 0.1 M phosphate buffer saline (PBS) and incubated in 3% v/v normal donkey serum (Jackson ImmunoResearch, Laboratories. Inc, West Grove, PA, USA) and 0.3% triton X-100 (Sigma-Aldrich, Madrid, Spain) for 2 h at room temperature. Sections were incubated at 4ºC overnight with the previous primary antibodies. They were then washed three times for 10 min in 0.1 M PBS, and incubated at room temperature for 2 h with a fluorescent secondary antibody, namely donkey anti-rabbit IgG Alexa Fluor 488 (1:500; RD systems, Barcelona, Spain). Finally, sections were mounted on slides with a fluorescence mounting medium composed of Mowiol 40–88 (Sigma-Aldrich), 87% glycerol, water, and 2,5%1,4-diazabicyclo-[2.2.2]octane, and coverslipped for microscopy and photography.

5.3. Image analysis to evaluate immunofluorescence studies

Three images were taken of each brain structure bilaterally. Sample areas were visualized under a 20X or 40X objective in a Leica DMR microscope, and digitized using a Leica DFC 300 FX digital camera (Vashaw Scientific Inc, Atlanta, USA). ImageJ software was used for the quantification of glial cells. To evaluate microglia activation, samples stained with Iba1 were selected. The background was subtracted by adjusting the detection threshold density, and we only considered the signal density above the threshold. Astrocytes were analysed by counting GFAP immunoreactive cells. The investigator was blind to the groups analysed. The number and percentage of stained pixels per area was measured automatically, and the average of each sample was calculated.

6. Tryptophan-kynurenine pathway analysis

We evaluated the TRP-KYN pathway in a distinct group of animals from those used for the behavioural experiments. We quantified the following products of this metabolic pathway in PFC and HC samples from adolescent mice: TRP, 5-HT, 5-hydroxyindolacetic acid, KYN, kynurenic acid, 3-hydroxykynurenine and xanthurenic acid (See Fig. 2 Introduction Section).

6.1. Chemicals and reagents

TRP, 5-HT, 5-hydroxyindoleacetic acid, KYN, kynurenic acid, 3-hydroxykynurenine, xanthurenic acid, and ammonium formate (High-performance liquid chromatography (HPLC) grade) were obtained from Sigma-Aldrich (St Louis, MO, USA), ritalinic acid from Steraloids Inc. (Newport, RI, USA), and formic acid (liquid chromatography-tandem mass spectrometry (LC-MS/MS) grade) and methanol (LC gradient grade) from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

6.2. Quantification of metabolites by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

At PD30, naïve mice were sacrificed by decapitation, the brain was rapidly removed, and the PFC and the HC were dissected using a brain tissue blocker. All samples were immediately frozen in dry ice and stored at -80ºC. For tissue processing, 500 µl of ice-cold buffer (0.5 mN sodium metabisulfate, 0.2 N perchloric acid and 0.5 mM ethylenediaminetetraacetic acid (EDTA)) (Biskup et al. 2012) and 30 µl of the internal standard solution (ritalinic acid 1 µg/mL in methanol:water (1:1) with 20 mM ascorbic acid) were added to the tissue, which was then

homogenized using a sonicator. Samples were centrifuged for 10 min (10.000 g at 4ºC) and the supernatant was kept on ice until analysis. 75 µL of extract were diluted with 75 µL of water for quantification of TRP, 5-HT, 5-hydroxyindoleacetic acid and KYN. 500 µL of extract were evaporated under nitrogen at 20ºC for quantification of kynurenic acid, 3-hydroxykynurenine and xanthurenic acid. The dry residue was dissolved in 75 µL of 0.5 M acetic acid. 20 µL of extract were injected into the LC-MS/MS system, which consisted of a triple quadrupole (Xevo) mass spectrometer (Waters Associates, Milford, MA, USA) coupled to an Acquity UPLC system (Waters Associates) for chromatographic separation. LC separation was performed using an Acquity BEH C_{18} column (10 cm × 0.21 cm, inner diameter, 1.7 µm) (Waters Associates), at a flow rate of 300 µL min^{-1}. The mobile phase solvents were water and methanol, each containing 0.01% v/v formic acid and 1 mM ammonium formate. The gradient and Selected Reaction Monitoring (SRM) method are detailed in table 1. Analyte quantification was based on the integral of the analyte and internal standard peaks, and a calibration curve constructed.

Table 1. SRM method. Analytes were determined using an SRM method by acquiring two transitions for each compound. The most specific transition was selected for quantitative purposes. Data were managed using the MassLynx software.

Analyte	MW	Ionization	Precursor m/z	Cone voltage (V)	Collision energy (eV)	Product m/z
TRP	204	ESI+	205	15	10	188*
5-HT	176	ESI+	177	10	5	160*
5-HIAA	191	ESI+	192	25	20	146*
KYN	208	ESI+	209	15	10	192*
3-HK	224	ESI+	225	15	10	208*
KA	189	ESI+	190	20	20	144*
XA	205	ESI+	206	20	20	160*

Table 1. Molecular weight (MW) and SRM conditions for the analytes studied (* transition used for quantification). Tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA).

7. Studies to evaluate rewarding and addictive effects induced by cocaine

In addition, a different group of mice were used to carry out the cocaine conditioned place preference, cocaine-induced sensitization and cocaine self-administration experiments. In this study, only male mice were evaluated to simplify the experimental design.

7.1. Drug and injection procedure

Cocaine was obtained from Agencia del Medicamento (Ministery of Health, Spain) and was dissolved in sterile physiology saline (0,9%). For the conditioned place preference paradigm (CPP) experiments, cocaine was administered by intraperitoneal route (i.p.) at the doses of 1.5, 3, 15

and 25 mg/kg. For locomotor sensitization study, cocaine was injected at the dose of 7.5 mg/kg (i.p.). For the self-administration procedure, cocaine was administered at the dose of 1mg/kg per infusion by intravenous (i.v.) route.

7.2 Conditioned Place Preference Paradigm

7.2.1. Procedure

The CPP is an experimental behavioural model used to assess the motivational properties of drugs. This task involves a classical conditioning learning process in which animals perform an association of a particular environment with the psychotropic effects of a drug treatment, whereas a second and different environment will be associated with the absence of the drug (vehicle-paired compartment) (Valverde et al. 1996; Roux et al. 2001; Prus et al. 2009).

In adolescent male mice, the rewarding properties of cocaine (1.5, 3, 15 and 25 mg/kg, i.p.) were evaluated using the CPP paradigm, as previously described (Valverde et al. 1996; Matthes et al. 1996). The CPP was carried out in a box divided in two different compartments consisting in two square chambers (20 x 20 x 20 cm) connected by a triangular grey compartment (20 x 20 x 20 cm). The two compartments differed in colours and floor textures. One of the compartments was black with a black rough textured floor and the other compartment was white with a transparent plastic floor with black horizontal lines. A grey neutral section separated the two chambers (Fig. 14A). The procedure was conducted in three different phases, as follow (Fig. 14B):

Pre-conditioning Phase. The first phase consisted of 20 min of free exploration of the box. For that, each mouse was placed in the middle of

the neutral area and was allowed to explore the both compartments. Mice showing preference or aversion for one of the compartments (more than 70% or minus than 30%) were excluded from the experiment.

Conditioning Phase. For the next six days, the mice were injected with cocaine or saline in alternate days. Mice received cocaine on days 1, 3 and 5 and received saline on days 2, 4 and 6 of the Conditioning Phase. Control animals received saline every day. In all the cases, immediately after the injection, each mouse was immediately placed into the assigned compartment during 30 min.

Testing Phase. On the eighth day, mice were allowed to explore both compartments for 20 minutes, similar to the pre-conditioning phase. The time spent in each compartment was calculated. A score was calculated as the difference of time spent in the compartment associated to the drug on the post-conditioning and the pre-conditioning.

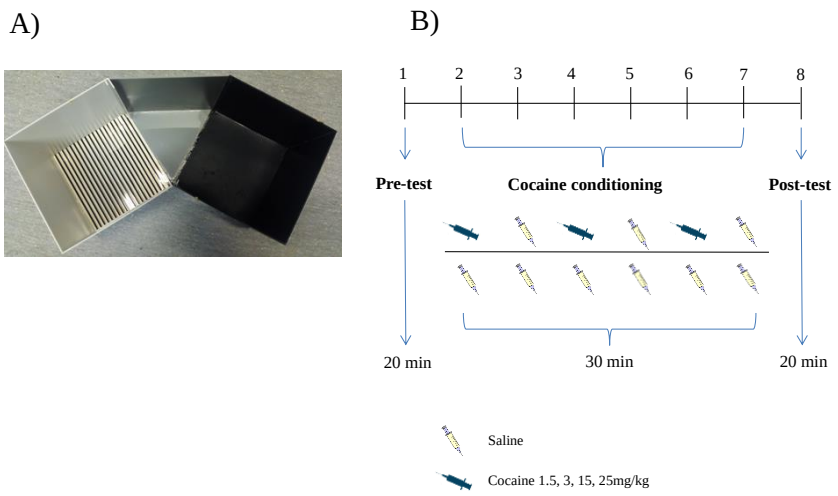


Figure 14. A) Conditioned place preference box. B) Schematic representation of the schedule used for cocaine conditioned place preference procedure in mice.

7.2.2. Preparation of tissue extract

Mice were sacrificed by decapitation 1 h after the test. The brains were rapidly removed and stored immediately at -80ºC until use for western blot analysis (DAT, DR2, Nurr1 and Pitx3) and HPLC analysis (DA and its metabolite DOPAC levels). Brains were sliced on a cryostat and kept at -20ºC until each region of interest comes into the cutting plane. NAc and VTA were micro-punched from frozen brain sections (500 µm), sectioned using a cryostat, according to the mice brain atlas (Paxinos and Franklin 2004). All micro-punched samples were stored frozen at -80ºC.

7.2.3. Dopaminergic system evaluation: Electrophoresis and Western Blotting

As previously described (García-Pérez et al. 2014; García-Pérez et al. 2015a), bilateral punches from NAc and VTA were placed in a buffer containing PBS, 10% sodium dodecyl sulphate (SDS), protease inhibitors (Boehringer Mannheim, Mannheim, Germany) and a phosphatase inhibitor Cocktail Set (Calbiochem, Darmstadt, Germany), homogenized and sonicated for 30 s before centrifugation at 6.000 g for 10 min at 4°C. Samples containing 20 µg of protein were loaded on a 10% SDS/polyacrylamide gel, electrophoresed and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Nonspecific binding of antibodies was prevented by incubating the membranes in 1% bovine serum albumin in Tris-buffered saline Tween-20 (10 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.15% Tween 20). The blots were incubated at 4ºC overnight with the following primary antibodies: rabbit polyclonal anti-Nurr1 (1:500; sc-991, Santa Cruz Biotechnology, Santa Cruz, CA,USA); rabbit polyclonal anti-Pitx3 (1:750; ab30734, Abcam, Cambridge, UK), rat monoclonal anti-DAT (1:2000; MAB369, Millipore) and rabbit polyclonal anti D2R (1:500;AB5084P, Millipore).

Goat anti-rabbit immunoglobulin G (IgG), Horseradish peroxidase (HRP)-linked (1:5000; sc-2004, Santa Cruz, Biotechnology) or goat anti-rat IgG, HRP-linked (1:5000;sc-2032, Santa Cruz Biotechnology) were used as secondary antibodies. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, Little Chalfont, Buckinghamshire, UK9 and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Blots were incubated with stripping buffer (glycine 25mM, SDS 1%, pH 2) for 1 h at 37ºC and subsequently reblocked and probed with rabbit polyclonal antiglyceraldehyde 3-phosphate dehydrogenase (GADPH) (Cell Signaling Technology Inc., Danvers, MA, USA) or α-tubulin (Cell Signaling Technology Inc.), which were used as loading control. The ratios DAT/GADPH, D2R/GADPH, Nurr1/α-tubulin and Pitx3/α-tubulin were plotted and analysed. Proteins levels were corrected for individual levels

7.2.4. Estimation of DA and its metabolite DOPAC

DA and DOPAC were determined in the NAc by HPLC with electrochemical detection, as previously described (García-Pérez et al. 2015b). One punch from each animal was obtained and added to 60 µl of a solution composed by 1M perchloric acid (Sigma Chemical Co) and 2.7 mM EDTA (Sigma Chemical Co). The samples were homogenized by slight sonication for about 1 min, centrifuged (6000 g; 4ºC) for 10 min and the supernatants were taken for analysis and filtered through ultra-free MC 0.2 µm filter (Millipore). The pellets were re-suspended by adding 100 µl of 1N sodium hydroxide. Then, the total amount of proteins from each sample was measured by spectrophotometry. From each sample 10 µl was injected into a 5-µm C18 reversed-phase column (Waters, Milford, MA, USA) through a Rheodyne syringe-loading injector 200 µL loop

(Waters). The mobile phase consisted of a 95% (v/v) mixture of water and methanol with sodium acetate (50 mM) (Sigma Chemical Co), citric acid (20 mM) (Sigma Chemical Co), L-octyl-sodium sulfonate (3.75 mM) (Sigma Chemical Co), di-n-butylamine (1 mM) (Sigma Chemical Co) and EDTA (0.135 mM) (Sigma Chemical Co), adjusted to pH 4.3. Chromatographic data were analysed with Millenium 2010 Chromatography Manager Equipment (Millipore). DA and DOPAC was simultaneously detected and quantified by reference to calibration curves run at the beginning of the assays. The content of DA and DOPAC is expressed as ng/mg of protein. The DA turnover was determined as the DOPAC/DA ratio. The DOPAC/DA ratio was used as an index of transmitter metabolism.

7.3. Cocaine-induced locomotor sensitization procedure

Repeated administration of psychostimulants in rodents produced a progressive increase in drug-induced locomotor activity that is higher when compared to that induced by a single injection. This mechanism is called locomotor sensitization and induces an increase in the activation of DA system after the stimuli, producing a higher increment in DA neurotransmission (Robinson and Berridge 1993). Moreover, sensitization is related to compulsive patterns of drug-seeking behaviour (Robinson and Berridge 1993; Robinson and Berridge 2001). The enhancement in the locomotor activity is a long-lasting effect and persists after a drug-free period in response to a single dose (challenge) of the psychostimulant compound. This test allows assessing the potential abuse liability of a compound due to its properties to induce locomotor sensitization (Robinson and Berridge 1993; Paterson et al. 2010).

In our study, the sensitization to the hyperlocomotor response elicited by cocaine was evaluated in adolescent male mice accordingly to a procedure previously described (Blanco et al. 2011) with minor modifications (Fig. 15). To review locomotor activity boxes and room conditions see section *4.1. Spontaneous locomotor activity*.

The procedure was conducted as follows, on the first day, mice were handled and weighted. The following day, mice were injected with saline and their basal locomotor activities were recorded during 30 min. During the following five consecutive days, mice were daily injected with cocaine (7.5 mg/kg) or saline, and then, immediately placed in the locomotor activity boxes to record the locomotor activity during 30 min. After this repeated treatment with cocaine, mice remained in their home cage during five days without receiving any treatment. After this period of time, on day 13, mice received a saline injection and the possible conditioned response associated with the environment into the locomotor activity box was evaluated. On day 14, mice received a cocaine challenge (7.5 mg/kg, ip) and then, sensitization behaviour was investigated. However, only the first 15 min were analyzed.

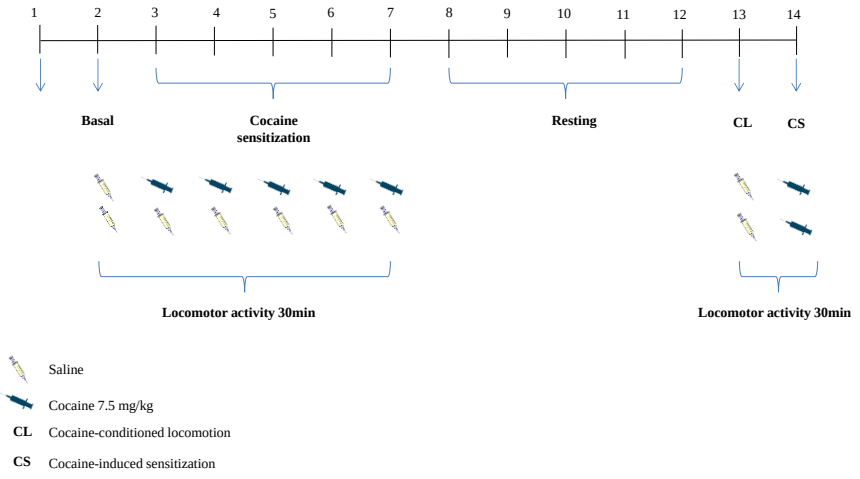


Figure 15. Schematic representation of the schedule used to investigate cocaine-induced locomotor sensitization in mice.

7.4. Operant cocaine self-administration

Intravenous (i.v.) drug self-administration procedure in rodents is used as a model for investigating the abuse liability of drugs of abuse using an experimental animal model, for studying the neurobiological basis of drug addiction, and for evaluating treatments for drug abuse, dependence and relapse (Thomsen and Caine 2005).

7.4.1. Surgery for the i.v. catheter implantation

At adolescence, mice were anesthetized with ketamine/xylazine (100 mg/kg, 20 mg/kg, respectively) and then implanted with indwelling i.v. silastic catheter as described previously (Soria et al. 2005). Briefly, a 4.5 cm length of silastic tubing (0.3 mm inner diameter, 0.64 mm outer diameter) (SilasticR, Dow Corning, Houdeng-Goegnies, Belgium) was adapted to a 22-gauge steel cannula (Semat; Herts; England) that was curved at a 90° angle and then placed in a cement disk (Dentalon plus, Heraeus Kulzer, Germany). The catheter tubing was inserted 1.4 cm into the right jugular vein (limited with a little dab of silicone) and held with suture. The rest of the catheter passed subcutaneously (s.c.) from the insertion position to the cannula that was fixed in the back of mice, this incision was closed with staples. All incisions were coated with Betadine (Meda Pharma SAU, San Fernando de Henares, Madrid, Spain). Mice were treated with analgesia (meloxicam; 0.5mg/kg; s.c.; Inflacam, Chanelle Ltd, Loughrea, Co., Galway, IE) and antibiotic (Enrofloxacino; 7.5 mg/kg; i.p.; Batryl, Bayer Hispania S.L, Sant Joan Despí, Barcelona, Spain) in order to improve the mice's recovery. After surgery, mice were

allowed to recover for 3-5 days prior to start the self-administration procedure.

7.4.2. Apparatus for self-administration experiments

The self-administration experiments were carried out in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Georgia, VT, USA) containing two holes; one was defined as active and the other as inactive. Nose-poking on the active hole produced a cocaine infusion (reinforce) that was paired with two light stimulus, one placed inside the nose-poke and the other above the hole. No consequences had nose-poking in the inactive hole. The side, in which active/inactive hole was placed, were counterbalance. The chambers were housed in sound- and-light- attenuated boxes provided with fans to provide ventilation and white noise (Fig. 16).

Figure 16. Self-administration chamber

7.4.3. Drug self-administration procedure

Cocaine self-administration session was performed as described previously (Soria et al. 2005; Soria et al. 2006; Martini and Valverde 2011; Tourino et al. 2012) with minor modifications. Responding was maintained by cocaine (1mg/kg per infusion) delivered in 20µl over 2s. Cocaine was infusion via a syringe that was set on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) and 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 to the mouse i.v. catheter. Self-administration sessions (1 h daily) were conducted during 10 consecutive days. At the beginning the chamber light was on during 3 seconds and off during the rest of the session. The session started with a priming infusion of the drug. Mice were trained under a fixed ratio 1 schedule of reinforcement. The number of reinforces was limited to 50 per session to avoid overdoses and each reinforce was followed by a 30 s time-out period where nose-poking in the active hole had no consequences. A mouse was considered to acquire when the number of responses in the active hole was at least 5, exceeded 75% of that on the inactive hole and it maintained a stable responding with less than 30% deviation from the mean of the total number of cocaine infusions obtained in two consecutive days (70% of stability). The patency of the i.v. catheters was evaluated at the end of the experiment by infusion of 0.1 ml of thiopental sodium (5 mg/ml; i.v.; B. Braun Medical, S.A. Rubí, Barcelona Spain). If signs of anaesthesia were not appear within 3 seconds, the mouse was removed from the experiment.

8. Statistical analysis

We compared the effects of maternal manipulation on maternal care using two-way ANOVA (group and day or group and period of the day), followed the Bonferroni post-hoc test. We evaluated the effects of rearing conditions on offspring body weight, behavioural performance, inflammatory response, and TRP-KYN pathway activity using a two-way ANOVA (group and sex), followed the Bonferroni post-hoc test. In the quantification of xanthurenic acid in the PFC, we use a one-way ANOVA (group effect). To analyse data obtained in the CPP experiments a two-

way ANOVA was calculated with rearing groups and treatment factors of variation, followed the Bonferroni post-hoc test. Western-blot and HPLC experiments were analyzed by two-way ANOVA calculated with group and treatment factors of variation followed by a post hoc Bonferroni test. For the cocaine locomotor sensitizacion studies, we calculated a three-way ANOVA with repeated measures (day factor) and between factors (rearing groups and treatment) followed by the Bonferroni post-hoc test. To analyse data from the 10-days training in the self-administration paradigm, a three-way ANOVA was calculated with repeated measures (day factor) and between factors (rearing groups and hole active/inactive) followed by Bonferroni post-hoc test. Data are represented as mean ± SEM. A p-value < 0.05 was considered statistically significant. Data were analyzed using SPSS v19.

RESULTS

1. Spontaneous maternal care evaluation

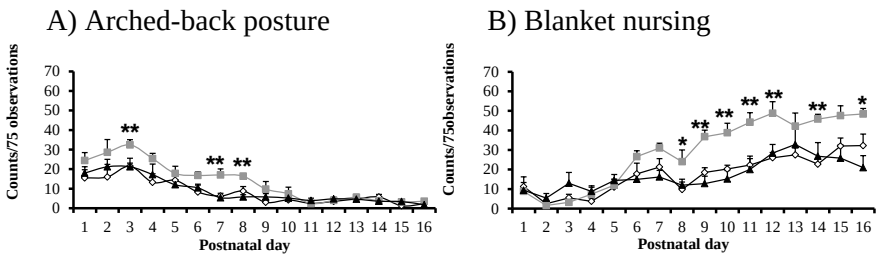
To study the effect of rearing conditions on maternal behaviour, spontaneous maternal care was recorded 3 times every day from PD1 to PD16. In the case of CN, we did not distinguished between biological and non-biological mothers due to the difficulty of pups to be identified. To evaluate maternal care, the following signs were observed and scored: 1) mother licking and grooming any offspring (body + anogenital region); 2) mother nursing offspring in an arched-back posture with rigid limbs ("high kyphosis"); 3) mother nursing in a "blanket" posture, i.e. lying on the offspring or her limbs are rigid but she has a low dorsal arch posture ("low/partial kyphosis"); 4) mother nursing in a "passive" posture ("supine nursing"), i.e. lying on her back or side while the offspring nurse; and 5) mother "off" offspring (no maternal contact).

Two-way ANOVA of ***arched-back nursing, blanket posture*** and ***off-nest behaviour*** during PD1 to PD16 revealed rearing group and day effects, and an interaction between these factors in blanket posture (Table 2). Bonferroni post-hoc analysis calculated for ***arched-back nursing*** (rearing conditions) showed significant differences in MSEW group when compared with SN group. These differences were found at PD3, PD7 and PD8 ($p<0.01$), indicating that MSEW mothers exhibited the arched-back posture more often than SN mothers (Fig. 17A). Post-hoc analysis calculated for ***blanket posture*** (rearing groups) presented significant differences in MSEW group when compared with SN group. These differences were found at PD8 ($p<0.05$), from PD9 to PD12 ($p<0.01$), PD14 ($p<0.01$), and PD16 ($p<0.05$), indicating that MSEW mothers presented the blanket posture more often than SN mothers (Fig. 17B). Post-hoc analysis for ***off-nest behaviour*** (rearing groups) showed significant differences in MSEW group when compared with SN group. These differences were found at PD4 ($p<0.01$) and from PD6 to PD10

($p<0.01$). These results showed that MSEW mothers spent less time out of the nest than SN mothers (Fig. 17C). However, no significant differences were found when CN group was compared with SN group. In addition, we observed no significant differences for other parameters related to maternal care.

Table 2. Two-way ANOVA for maternal care evaluated from PD1 to PD16. Rearing group (R), Day (D).

	Maternal care	
	Arched-back nursing	
	F	**P <**
R	F(2,11)=14.098	0.01
D	F(15,165)=22.900	0.01
RxD	F(30,165)=1.125	NS
	Blanket posture	
	F	**P <**
R	F(2,11)=15.424	0.01
D	F(15,165)=21.907	0.01
RxD	F(30,165)=2.655	0.01
	Out off nest	
	F	**P <**
R	F(2,11)=21.443	0.01
D	F(15,165)=13.941	0.01
RxD	F(30,165)=1.395	NS



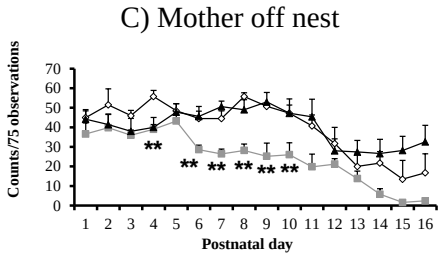
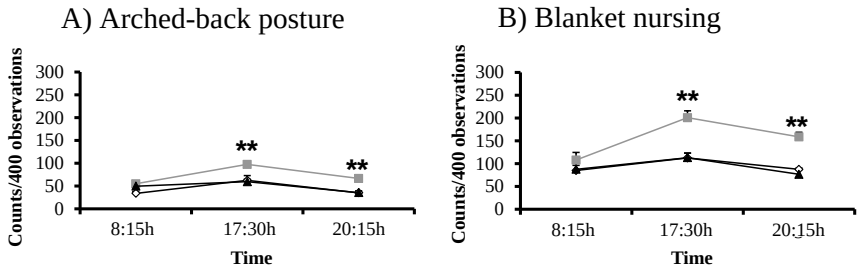


Figure 17. Effects of rearing conditions on maternal behaviour during the first 16 PD. Maternal behaviours were evaluated three times per day from PD1 to 16 (8:15 h , 17:30 h and 20:15 h): arched-back posture (Panel A), blanket nursing (Panel B) and off-nest behaviour (Panel C). White symbols represent SN group of mice; Grey symbols represent MSEW group of mice; Black symbols represent CN group of mice. Data are expressed as the mean daily (± SEM) count (75 observations/day) of each behaviour, N=4-6 mothers per group. * $p<0.05$; ** $p<0.01$ SN vs. MSEW group.

As reported in the Methodology Section, maternal care was analysed three times per day (8:15 h, 17:30 h and 20:15 h). Two-way ANOVA of ***arched-back nursing, blanket posture*** and ***off-nest behaviour*** revealed rearing group and period of the day effect, and an interaction between these factors (Table 3). Bonferroni post-hoc analysis calculated for ***arched-back nursing, blanket posture*** and ***off-nest behaviour*** (rearing conditions) revealed significant differences in MSEW group when compared with SN group at 17:30 h and 20:15 h ($p<0.01$) (Fig. 18), showing that MSEW mothers displayed an enhanced maternal care when compared with SN. Nevertheless, the maternal care of CN mothers was similar to SN mothers.

Table 3. Two-way ANOVA for maternal care evaluated at three different period of the day (8:15 h, 17:30 h and 20:15 h). Rearing group (R), Period of the Day (P).

	Maternal care	
	Arched-back nursing	
	F	**P <**
R	F(2,11)=23.880	0.01
P	F(2,22)=23.232	0.01
RxP	F(4,22)=2.558	0.05
	Blanket posture	
	F	**P <**
R	F(2,11)=27.144	0.01
P	F(2,22)=12.560	0.01
RxP	F(4,22)=3.068	0.05
	Out off nest	
	F	**P <**
R	F(2,11)=34.214	0.01
P	F(2,22)=18.858	0.01
RxP	F(4,22)=4.644	0.01



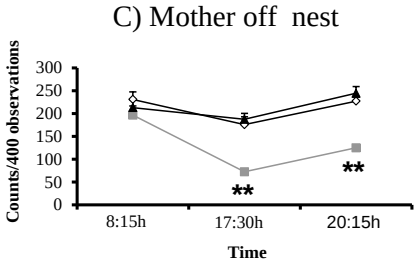


Figure 18. Effects of rearing conditions on maternal behaviour on different periods of the day. Maternal behaviours were evaluated three times per day at 8:15 h, 17:30 h and 20:15 h during the first 16 PD: arched-back posture (Panel A), blanket nursing (Panel B) and off-nest behaviour (Panel C). White symbols represent SN group of mice; Grey symbols represent MSEW group of mice; Black symbols represent CN group of mice. Data are expressed as the mean daily (± SEM) count (400 observations during the first 16 PD) of each behaviour, N=4-6 mothers per group. ** $p<0.01$ SN vs. MSEW group.

2. Effects of rearing conditions on body weight

Body weight was measured in a group of mice at PD10, 17, 30, 62 and 83. Significant differences were observed between rearing groups at PD10 and PD17. Two-way ANOVA calculated for body weight at PD10 and PD17 showed a rearing group effect ($F(2,81)=36.042$; $p<0.01$) and ($F(2,81)=131.066$; $p<0.01$), sex effect ($F(1,81)=32.408$; $p<0.01$) and ($F(1,81)=40.057$; $p<0.01$), without interaction between both factors ($F(2,81)=0.771$; NS) and ($F(2,81)=0.440$; NS), respectively. Bonferroni post-hoc analysis showed that mice from MSEW group had lower body weight than mice from SN in male and female ($p<0.01$). Moreover in CN group, male showed higher body weight at PD17 and female at PD10 and PD17 when compared with SN group ($p<0.01$). Finally, male mice exhibited higher body weight than female in SN, MSEW and CN group ($p<0.01$) (Fig. 19).

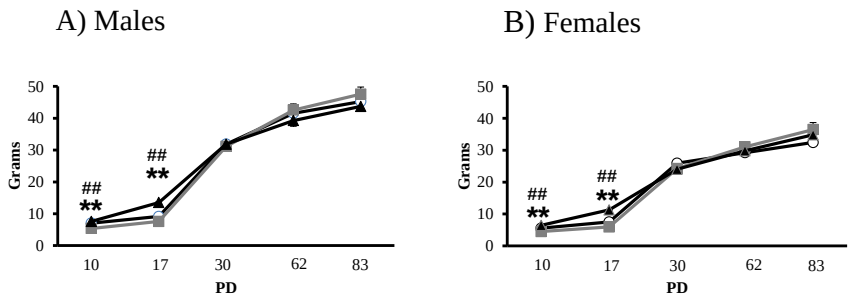


Figure 19. Effects of rearing conditions on body weight at different postnatal day. Body weight was evaluated at PD10, 17, 30, 62 and 83 in males (A) and females (B). White symbols represent SN group of mice; Grey symbols represent MSEW group of mice; Black symbols represent CN group of mice. Data are expressed as the body weight (grams) (± SEM), N=10-17 mice per group. ** $p<0.01$ SN vs. MSEW group. ## $p<0.01$ SN vs. CN group.

3. Effects of rearing conditions on the emotional behaviour

3.1. Effects of rearing conditions on locomotor activity

Spontaneous locomotor activity was evaluated in both adolescent (PD31-33; Fig. 20A and B) and in adult mice (PD91-93, Fig. 20C and D). In adolescent mice, two-way ANOVA for ***horizontal activity*** (deambulations) showed a rearing group effect ($F(2,104)=3.728$; $p<0.05$), but no sex effect ($F(1,104)=0.006$; NS), and no interaction between these two factors ($F(2,104)=0.626$; NS). Bonferroni post-hoc test calculated for horizontal activity showed that male mice from the MSEW group exhibited lower horizontal activity than those from the SN group ($p<0.05$; Fig. 20A). Two-way ANOVA of ***vertical activity*** (rearing) also showed a significant rearing group effect ($F(2,104)=5.729$; $p<0.01$), but no sex effect ($F(1,104)=3.681$; NS), and no interaction between these two factors ($F(2,104)=0.109$; NS). Post-hoc analysis indicated that male mice from the MSEW group presented a lower vertical activity than those from the

SN group ($p<0.05$). No differences were found between CN group and SN group and in female mice (Fig. 20B).

We also evaluated the spontaneous locomotor activity at adulthood (PD91-93), but we observed no significant differences (Fig. 20C and D). Two-way ANOVA for ***horizontal activity*** (deambulations) showed no rearing group effect ($F(2,101)=0.35$; NS), no sex effect ($F(1,101)=1.462$; NS), and no interaction between these two factors ($F(2,101)=2.372$; NS) (Fig. 20C). Two-way ANOVA of ***vertical activity*** (rearing) showed no rearing group effect ($F(2,101)=1.789$; NS), no sex effect ($F(1,101)=1,953$; NS), and no interaction between these two factors ($F(2,101)=0.108$; NS) (Fig. 20D).

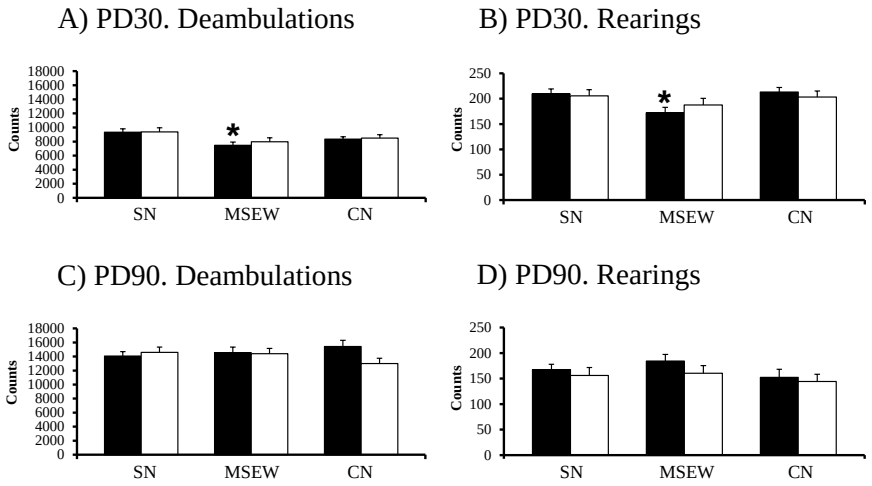


Figure 20. Effects of rearing conditions on spontaneous locomotor activity among offspring. Horizontal activity (deambulations) (A and C) and vertical activity (rearings) (B and D) were evaluated in male and female adolescent offspring on PD31-33 and at adulthood, PD91-93. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean (± SEM) of photocell counts during a 20 min period. N=14-23 mice per group at PD31-33 and N=14-18 at PD91-93. * $p<0.05$ vs. SN group.

3.2. Effect of rearing conditions on anxiety-like behaviour in the elevated plus maze

Mice were exposed to the EPM during adolescence, at PD32-35, and in adulthood, at PD92-95.

For adolescent mice (PD32-35), two-way ANOVA calculated for ***the percentage time spent in open arms*** showed a rearing group effect ($F(2,90)=7.457$; $p<0.01$), but no sex effect ($F(1,90)=0.046$; NS), and no interaction between these two factors ($F(2,90)=0.239$; NS) (Fig. 21A). Bonferroni post-hoc test showed lower percentage of time spent in the open arms in the MSEW group than in the SN group for both males ($p<0.05$) and females ($p<0.01$) mice. Unexpectedly, CN female mice spent less time in open arms when compared with SN female mice ($p<0.05$) (Fig. 21A). Two-way ANOVA calculated for the ***percentage of entries into the open arms*** of the maze showed a rearing group effect ($F(2,90)=10.873$, $p< 0.01$), but no sex effect ($F(1,90)=0.048$; NS), and no interaction between these two factors ($F(2,90)=0.458$; NS) were found (Fig. 21B). Post-hoc analysis showed that mice from the MSEW group made significantly less entries into the open arms of the maze than mice from the SN group (in both males and females, $p<0.01$). Surprisingly, CN female mice also made less entries to the open arms when compared with SN female group ($p<0.05$) (Fig. 21B). We observed no differences in the ***total number of entries into the maze***, indicating that there was no significant difference in general activity between the three experimental groups in this model. Two-way ANOVA revealed no rearing group ($F(2,90)=0.595$; NS), no sex effect ($F(1,90)=0.004$; NS), and no interaction between both factors ($F(2,90)=1.137$; NS) (Fig. 21C).

We evaluated the response in the EPM during adulthood (PD92-95). Our results indicated that females were more sensitive to the deleterious

effects of maternal separation in this model. Hence, these effects persisted into adulthood. Two-way ANOVA for the ***percentage time spent in open arms*** showed a significant rearing group effect ($F(2,83)=7.215$; $p<0.01$), but no sex effect ($F(1,83)=0.051$; NS) and no interaction between these two factors ($F(2,83)=3.003$; NS) (Fig. 21D). Bonferroni post-hoc analysis showed that female adult mice from the MSEW and CN group exhibited a higher anxiety-like responses that those from the SN group ($p<0.01$). Two-way ANOVA for the ***percentage of entries into the open arms*** of the maze showed a rearing group effect ($F(2,83)=14.048$, $p< 0.01$), without sex effect ($F(1,83)=0.097$, NS), but interaction between these factors ($F(2,83)=7.897$; $p<0.01$). MSEW and CN female mice showed less number of entries into open arms than SN mice ($p<0.01$) (Fig. 21E). Two-way ANOVA calculated in the ***total number of entries into the maze*** revealed a rearing group effect ($F(2,83)=4.738$; $p<0.05$), but no sex effect ($F(1,83)=1.685$; NS) and no interaction between these factors ($F(2,83)=1.331$; NS) (Fig. 21F). Ulterior post-hoc analysis indicated that CN female mice made significant less total entries than SN female mice ($p<0.05$).

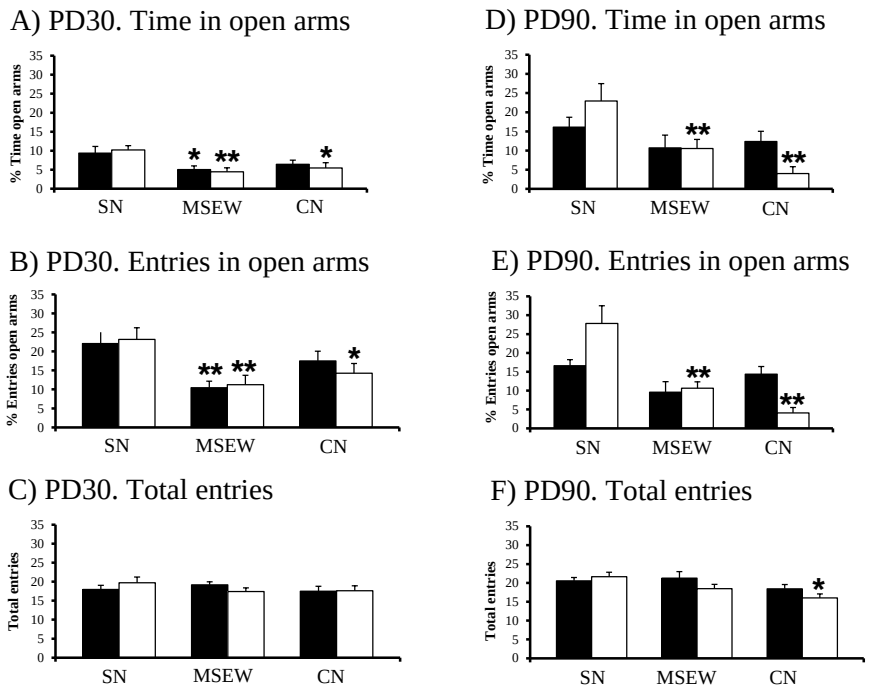


Figure 21. Effects of rearing conditions on the elevated plus maze. The percentage time spent in open arms (Panels A and D), the percentage of entries into open arms (Panels B and E) and the number of total entries (Panels C and F) were assessed in male and female offspring on PD32-35 and PD92-95. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean ± SEM, N=14–19 mice per group at PD32-35, and N=13-16 mice per group at PD92-95. * $p<0.05$, ** $p<0.01$ vs. SN group.

3.3. Effect of rearing conditions on despair behaviour in the tail suspension test

The effects observed in the TST were evaluated in female and male adolescent mice (PD33-36) and at adulthood (PD93-96). Two-way ANOVA of the ***time spent immobile*** in adolescent mice showed a significant rearing group ($F(2,98)=13.078$; $p<0.01$) and sex effect ($F(1,98)=10.088$; $p<0.01$), but no interaction between these two factors ($F(2,98)=2.694$; NS). Bonferroni post-hoc test showed that MSEW mice

spent more time immobile than SN mice (in both males ($p<0.05$) and females ($p<0.01$)) (Fig. 5A). Surprisingly, CN male mice spent more time immobile than SN group ($p<0.05$). We also observed a significant sex effect. Post-hoc analysis showed a significant difference in the time spent immobile between males and females from the MSEW group ($p<0.01$) (Fig. 22A). Interestingly, the greater despair behaviour observed in the MSEW group persisted into adulthood (PD93-96) in both males and females. Consequently, two-way ANOVA revealed a significant rearing group effect ($F(2,95)=10.516$; $p<0.01$), but no sex effect ($F(1,95)=2.315$; NS), and no interaction between these factors ($F(2,95)=1.116$; NS). Subsequent post-hoc analysis showed that mice from the MSEW group spent more time immobile than those from the SN group, in both males ($p<0.01$) and females ($p<0.05$). Surprisingly, CN female group also spent more time immobile than female from SN group ($p<0.01$) (Fig. 22B).

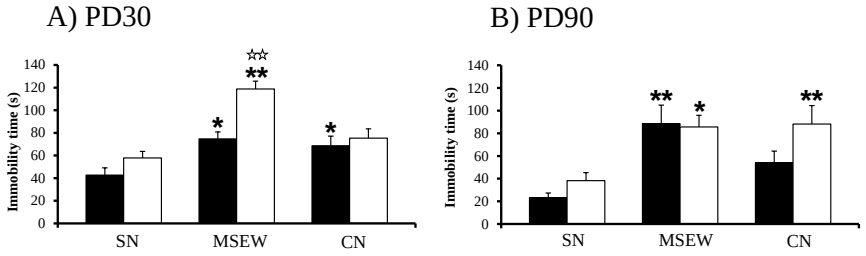


Figure 22. Effects of rearing conditions on the tail suspension test. Time spent immobile was evaluated in male and female offspring at PD33-36 (A) and PD93-96 (B). Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean ± SEM of time spent immobile (s), N=18-20 mice per group at PD33-36, and N=14-18 mice per group at PD93-96). * $p<0.05$, ** $p<0.01$ vs. SN group. Two white stars $p<0.01$ sex comparisons.

3.4. Effect of rearing conditions on anhedonia evaluated with the saccharin test

Saccharin test was performed in adolescent mice (PD34-40) (females and males) and at adulthood (PD94-100) to evaluate the anhedonia-like effects in mice. We evaluated saccharin preference over water and saccharin intake (g saccharin/Kg). At adolescent, two-way ANOVA for ***preference of saccharin at 24 h, 48 h*** and ***72 h*** revealed group effect (except at 48 h) and sex effect (except 24 h) without interaction between both factors (Table 4). Bonferroni post-hoc of ***saccharin preference at 24 h*** of group factor showed reduced saccharin preference in MSEW mice when compared with SN mice, in both male and female mice ($p<0.01$) (Fig. 23A). Post-hoc analysis of ***saccharin preference at 48 h*** indicated that males showed decreased saccharin preference when compared with females, in SN group ($p<0.01$), MSEW group ($p<0.05$), and CN group ($p<0.05$) (Fig. 23A). Bonferroni post-hoc analysis of ***saccharin preference at 72 h*** revealed that males showed reduced saccharin preference when compared with females, in SN group ($p<0.01$), MSEW group ($p<0.05$) and CN group ($p<0.01$) (Fig. 23A).

Two-way ANOVA calculated for ***g saccharin/Kg at 24 h, 48 h*** and ***72 h*** revealed group effect (except at 48 h) and sex effect (except 24 h) without interaction between both factors (Table 4). At 24 h, Bonferroni post-hoc analysis showed that MSEW female mice consumed less saccharin than female mice from SN group ($p<0.01$) (Fig. 23B). Post-hoc analysis also revealed that males consumed less saccharin than female mice, in SN, MSEW and CN groups at 48 h and 72 h ($p<0.01$) (Fig. 23B). Moreover at 72 h, Bonferroni post-hoc analysis showed than MSEW mice consumed less saccharin than mice from SN group, in both male ($p<0.05$) and female mice ($p<0.01$) (Fig. 23B). Surprisingly, CN male mice consumed less

saccharin that SN group ($p<0.05$). These results suggested that MSEW induced anhedonia in adolescent mice.

At adulthood (PD93-96), two-way ANOVA for ***preference of saccharin and g saccharin/Kg consumption at 24 h, 48 h*** and ***72 h*** revealed no significant differences between rearing groups, no sex effect and no interaction between both factors (Table 4).

Table 4. Two-way ANOVA calculated for saccharin test at adolescence and adulthood. Rearing group (R), Sex (S).

	% Saccharin preference					
	24h		**48h**		**72h**	
PD30	**F**	**P <**	**F**	**P <**	**F**	**P <**
R	F(2,47)=12.469	0.01	F(2,47)=1.139	NS	F(2,47)=3.082	0.05
S	F(1,47)=3.509	NS	F(1,47)=22.395	0.01	F(1,47)=21.352	0.01
RxS	F(2,47)=0.307	NS	F(2,47)=0.125	NS	F(2,47)=0.041	NS
	g Saccharin/kg					
	24h		**48h**		**72h**	
PD30	**F**	**P <**	**F**	**P <**	**F**	**P <**
R	F(2,47)=5.262	0.01	F(2,47)=2.085	NS	F(2,47)=7.244	0.01
S	F(1,47)=1.125	NS	F(1,47)=19.764	0.01	F(1,47)=20.206	0.01
RxS	F(2,47)=0,404	NS	F(2,47)=0.628	NS	F(2,47)=0.307	NS
	% Saccharin preference					
	24h		**48h**		**72h**	
PD90	**F**	**P <**	**F**	**P <**	**F**	**P <**
R	F(2,82)=2.528	NS	F(2,82)=0.611	NS	F(2,82)=1.179	NS
S	F(1,82)=0.251	NS	F(1,82)=11.904	NS	F(1,82)=0.072	NS
RxS	F(2,82)=0.692	NS	F(2,82)=0.539	NS	F(2,82)=0.604	NS
	g Saccharin/kg					
	24h		**48h**		**72h**	
PD90	**F**	**P <**	**F**	**P <**	**F**	**P <**
R	F(2,82)=1.980	NS	F(2,82)=1.773	NS	F(2,82)=1.382	NS
S	F(1,82)=1.236	NS	F(1,82)=1.558	NS	F(1,82)=2.493	NS
RxS	F(2,82)=0.627	NS	F(2,82)=0.022	NS	F(2,82)=0.377	NS

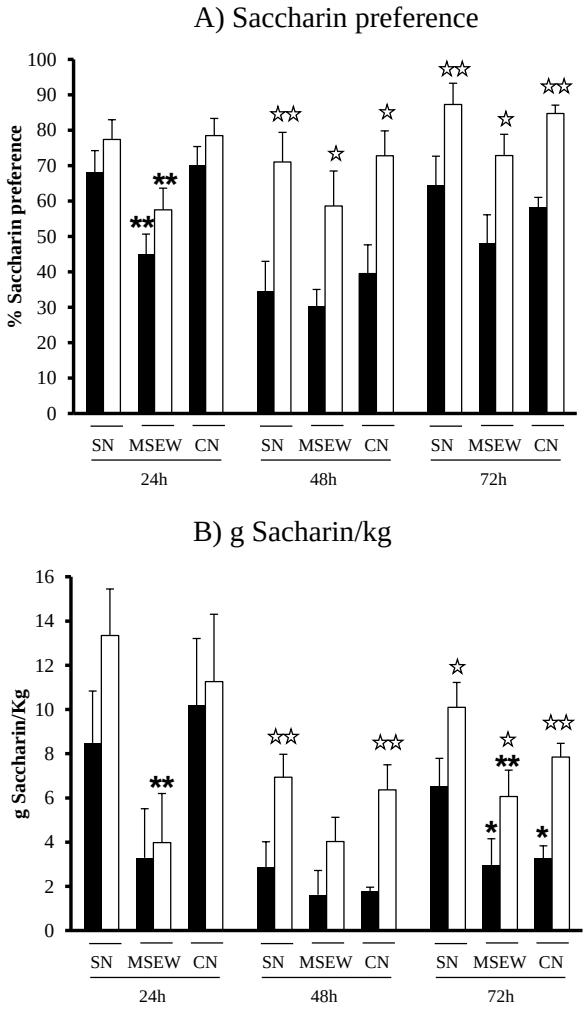


Figure 23. Effects of rearing conditions on the saccharin test at adolescence. Saccharin test was evaluated at PD34-40 in males and females mice. Saccharin preference (A) and g saccharin/kg intake (B) were calculated. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as mean ± SEM, N=7-8 mice per group. * $p<0.05$, ** $p<0.01$ vs. SN group. One white star $p<0.05$; two white stars $p<0.01$ sex comparisons.

3.5. Nociceptive threshold

As explained in Methodology Section, nociceptive threshold was assessed in SN and MSEW groups of mice. We evaluated the nociceptive threshold in mice by measuring the initial behavioural reactivity to electric foot-shock exposure in mice at PD30 (female and male) (Table 5). No significant differences were found between groups in the foot-shock reactivity (number of jumps and vocalisations), indicating a similar basal response. Two-way ANOVA for the ***number of jumps*** showed no rearing group effect (F(1,39)=0.382; NS), no sex effect (F(1,39)=0,266; NS), and no interaction between both factors (F(1,39)=0,005; NS) (Table 5). Moreover, no significant differences between rearing groups were found in the locomotor activity (number of rearings and squares crossed) during the habituation period. Two-way ANOVA for the ***number of rearing and squares crossed*** showed no rearing group effect (F(1,39)=0.510; NS) and (F(1,39)=4.088; NS), no sex effect (F(1,39)=0.110; NS) and (F(1,39)=0.985; NS), and no interaction between both factors (F(1,39)=2.082; NS) and (F(1,39)=1.105; NS), respectively) (Table 5).

Table 5. Effects of maternal separation on electric nociceptive threshold. Male (M), Female (F).

	Locomotor activity during habituation		**Electric foot shock reactivity**	
	Rearings	**Squares crossed**	**Jumping**	**Vocalisation**
SN M	25.7±4.36	80.3±8.34	30.2±2.94	10±0
SN F	30.3±5.35	101.2±4.45	28.2±2.94	10±0
MSEW M	29.9±2.51	94.2±5.64	28.5±3.41	10±0
MSEW F	26.9±1.71	100.8±7.97	26.9±2.23	10±0

Table 5. Electric foot-shock reactivity was evaluated in male and female offspring on PD30. Data are expressed as mean ± SEM of the number of behavioural parameters observed, N=10 mice per group. No significant differences were observed between groups in any behavioural parameters recorded.

3.6. Effect of rearing conditions on emotional memory evaluated in the passive avoidance paradigm

We conducted a passive avoidance test to evaluate differences in emotional memory (associative learning) at adolescence and adulthood. In adolescent mice (PD30-33) (females and males), we did not find differences in ***latency in acquisition trial*** for rearing group ($F(2,67)=0.984$; NS), no sex effect ($F(1,67)=0.981$; NS), and no interaction between both factors ($F(2,67)=1.450$; NS)**.** Two-way ANOVA of ***latency in retention trial*** showed a significant effect of rearing group ($F(2,67)=2.959$; $p<0.05$) and sex ($F(1,67)=11.768$; $p<0.01$), but no interaction between these two factors ($F(2,67)=0.891$; NS). Bonferroni post-hoc analysis showed that female MSEW mice exhibited shorter latency to enter the dark compartment than SN mice ($p<0.05$) associated with emotional memory impairments. No differences were observed between rearing groups in males (Fig. 24A). Therefore, there was significant difference between males and females in the response to retention trial in MSEW and CN group ($p<0.01$) showing that females had shorter latency to enter the black compartment than males (Fig. 24A). At adulthood (PD90-93), two-way ANOVA of ***latency in acquisition trial*** revealed significant effect of rearing group ($F(2,96)=5.587$, $p<0.01$), no sex effect ($F(1,96)=1.015$; NS), or interaction between these factors ($F(2,96)=0.059$; NS). Ulterior post-hoc analysis indicated that CN mice showed longer latency than SN group, in male and female mice ($p<0.05$) (Fig. 24B). In addition, two-way ANOVA of ***latency in retention trial*** showed a significant rearing group effect ($F(2,97)=6.996$; $p<0.01$), no sex effect ($F(1,97)=0.743$; NS), and interaction between these factors ($F(2,97)=6.636$; $p<0.05$). Bonferroni post-hoc test showed significant differences in retention trial between female. Therefore, female from MSEW and CN group exhibited shorter latency to enter the dark

compartment than SN female mice ($p<0.01$). These results confirmed that the impaired passive avoidance responses related to maternal separation persists into adulthood in female mice (Fig. 24B).

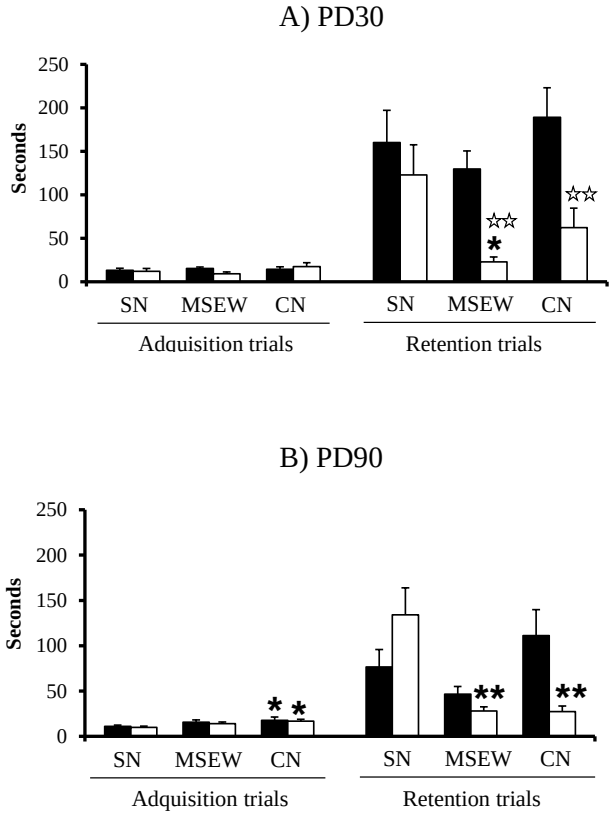


Figure 24. Effects of rearing conditions on the passive avoidance test. Latency time in entering the dark compartment in acquisition and retention trials in male and female offspring at PD30-33 (A) and PD90-93 (B) were evaluated. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as mean ± SEM of the latency time, N=10-12 mice per group at PD30-33, and N=14-17 mice per group at PD90-93. * $p<0.05$, ** $p<0.01$ vs. SN group. Two white stars $p<0.01$ sex comparisons.

4. Effect of rearing conditions on inflammatory reactions

4.1. Peripheral inflammatory response. Serum level of the cytokine IL-6

We determined the level of the cytokine IL-6 in serum of male and female adolescent mice in order to evaluate the peripheral inflammatory response. Two-way ANOVA of ***serum level of IL-6*** showed no group ($F(2,30)=0.818$; NS), no sex effect ($F(1,30)=0.479$; NS), and no interaction between these factors ($F(2,30)=0.995$; NS) (Fig. 25).

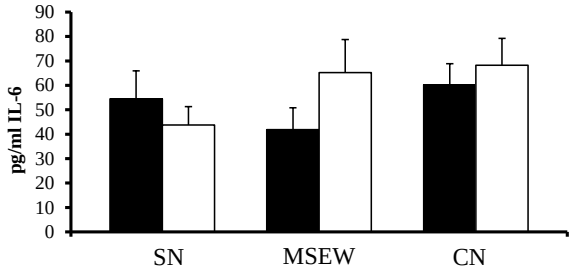


Figure 25. Effects of rearing conditions on serum level of IL-6. The serum level of IL-6 was assessed in male and female offspring at PD30. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as mean of the pg/ml ± SEM, N=4-7 mice per group.

4.2. Neuroinflammatory reactions in discrete brain areas

4.2.1. Microglia activation. Iba1 immunostaining

Microglia activation was evaluated using Iba1 staining in the PFC, and the CA1, CA3 and DG regions of the HC in adolescent mice (PD30). Two-way ANOVA of ***the percentage of stained area in the PFC*** showed a rearing group effect ($F(2,23)=5.081$; $p<0.05$), no sex effect ($F(1,23)=2.358$; NS), no interaction between these factors ($F(2,23)=1.053$;

NS). Bonferroni post-hoc analysis showed that female mice from MSEW and CN groups had greater stained area than female SN mice ($p<0.01$ and $p<0.05$, respectively) (Fig. 26A). We found no differences between rearing groups in male mice (Fig. 26A). For the ***hippocampal CA1 region***, two-way ANOVA showed a rearing group ($F(2,23)=13.665$; $p<0.01$) and sex effect ($F(1,23)=11.032$; $p<0.01$), and interaction between these factors ($F(2,23)=4.994$; $p<0.05$). Subsequent post-hoc analysis showed that female MSEW mice had a higher percentage of stained area than SN ($p<0.01$; Fig. 26B). Moreover, female CN mice had a higher percentage of stained area than SN mice ($p<0.01$). We also observed a significant difference between male and female MSEW group of mice ($p<0.01$), indicating that female mice had greater microglia activation than male mice (Fig. 26B). For the ***hippocampal CA3 region,*** two-way ANOVA showed a significant rearing group effect ($F(2,23)=3.947$; $p<0.05$), but no sex effect ($F(1,23)=0.127$; NS), and no interaction between these factors ($F(2,23)=1.159$; NS). Post-hoc analysis showed that female MSEW mice had higher microglia activation than female SN mice ($p<0.01$) (Fig. 26C). We observed no such differences in males. Finally, for the ***hippocampal DG region,*** two-way ANOVA of microglia activation showed no rearing group effect ($F(2,23)=2.380$; NS), sex effect ($F(1,23)=13.418$; $p<0.01$), but no interaction between these factors ($F(2,23)=0.967$; NS). Bonferroni post-hoc analysis showed that in MSEW and CN groups, female mice presented a higher percentage of stained area than male mice ($p<0.01$ and $p<0.05$, respectively) (Fig. 26D).

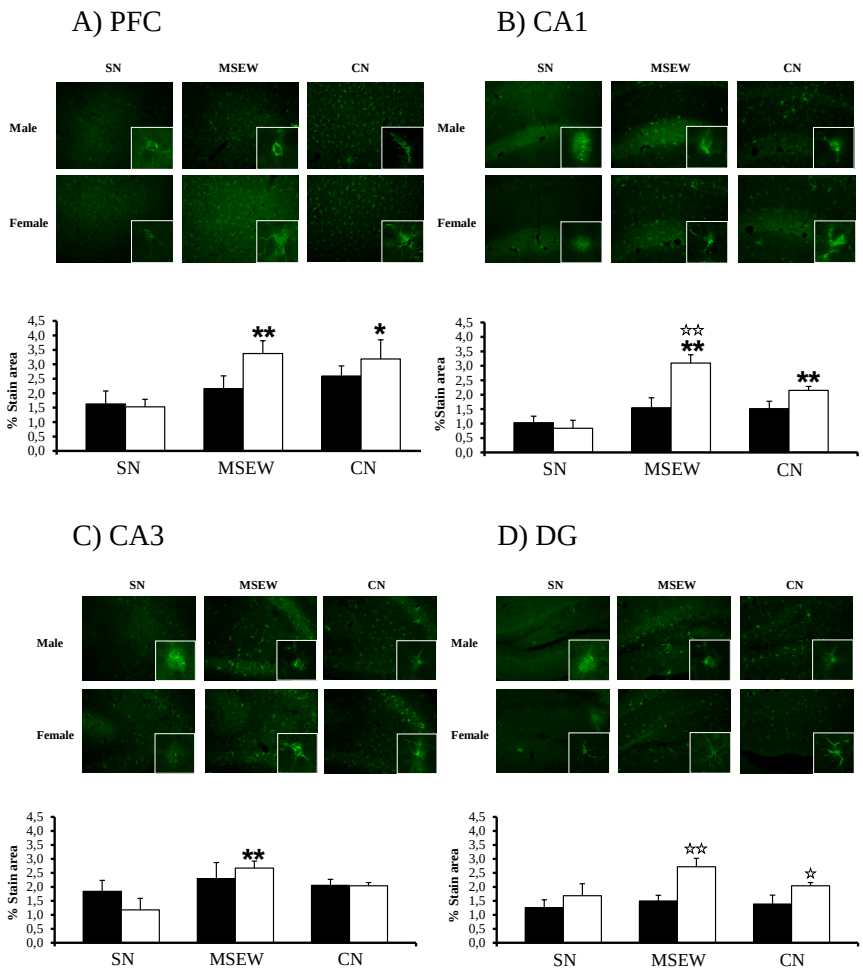
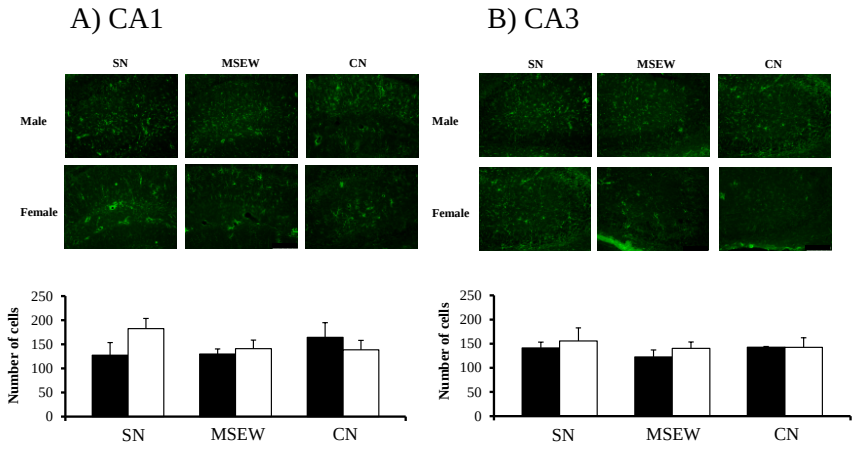


Figure 26. **Effects of rearing conditions on Iba1 staining.** Microglia activation was evaluated using Iba1 in the PFC (A), CA1 (B), CA3 (C), and DG (D) regions of the HC in SN, MSEW and CN mice at PD30. Black bars represent male groups of mice and white bars represent female groups of mice. Data of microglia staining quantification are expressed as mean of the percentage of stain area ± SEM, N=4 mice per group. ** $p<0.01$ vs. SN group. One white star $p<0.05$, two white star $p<0.01$, sex comparisons.

4.2.2. Astrocytes. GFAP immunostaining

Astrocytes were assessed using GFAP staining in the CA1, CA3 and DG regions of the HC; however, no significant differences were found. In adolescent mice (PD30), for the ***hippocampal CA1 region***, two-way ANOVA of astrocytes positive cells showed no rearing group effect ($F(2,23)=0.749$; NS), no sex effect ($F(1,23)=0.680$; NS), no interaction between these factors ($F(2,23)=0.175$; NS) (Fig. 27A). For the ***hippocampal CA3 region,*** two-way ANOVA indicated no rearing group effect ($F(2,23)=1.083$; NS), no sex effect ($F(1,23)=0.495$; NS), and no interaction between these factors ($F(2,23)=1.699$; NS) (Fig. 27B). Finally, for the ***hippocampal DG region,*** two-way ANOVA showed no rearing group effect ($F(2,23)=0.590$; NS), no sex effect ($F(1,23)=1.301$; NS), no interaction between these factors ($F(2,23)=0.922$; NS) (Fig. 27C).



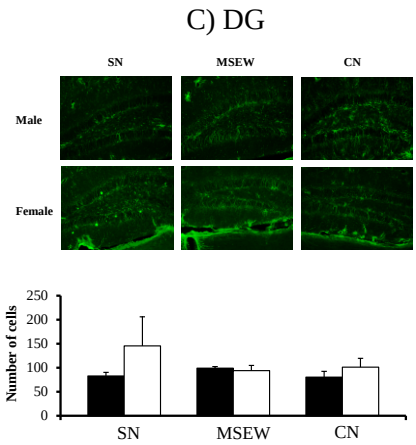


Figure 27. .Effects of rearing conditions on GFAP staining. Astrocytes were evaluated using GFAP staining in the CA1 (A), CA3 (B), and DG (C) regions of the HC in SN, MSEW and CN mice at PD30. Black bars represent male groups of mice and white bars represent female groups of mice. Data of astrocytes quantification are expressed as mean of the number of cells ± SEM, N=4 mice per group.

5. Rearing conditions produces alterations in the tryptophan-kynurenine metabolic pathway

To assess whether TRP-KYN metabolism was altered in the PFC and HC, representative brain areas that receive dense 5-HT innervation and play an important role in behavioural and emotional responses (Palazidou, 2012), we evaluated the TRP-KYN pathway in a distinct group of animals from those used for the behavioural experiments. We quantified the following products of this metabolic pathway in PFC and HC samples from adolescent mice: TRP, 5-HT, 5-hydroxyindolacetic acid, KYN, kynurenic acid, 3-hydroxykynurenine and xanthurenic acid. To evaluate the activity of the main enzymes involved, we also calculated the ratios between precursors and metabolic products of the TRP-5HT and TRP-KYN pathways. In the ***PFC***, analysis of ***TRP*** levels showed no rearing group

effect (F(2,47)=0.417; NS), no sex effect (F(1,47)=4.228; NS), no interaction between these factors (F(2,47)=0.530; NS). Two-way ANOVA of ***5-HT*** levels revealed no rearing group effect (F(2,47)=2.971; NS), sex effect (F(1,47)=8.803; p<0.01), and no interaction between these factors (F(2,47)=0.587; NS). Analysis of the ***5-hydroxyindolacetic acid*** indicated no rearing group effect (F(2,47=3.311; NS), without sex effect (F(1,47=47.299; NS) and no interaction between these factors (F(2,47=2.588; NS). Two-way ANOVA of the ***TRP/5-HT*** ratio showed no rearing group effect (F(2,47)=2.728; NS); no sex effect (F(1,47)=29.517; NS), and no interaction between both factors (F(2,47)=2.211; NS). Analysis of the ***5-hydroxyindolacetic acid/5-HT*** ratio showed a rearing group (F(2,47)=7.769; p<0.01) and sex effect (F(1,47)=45.338; p<0.01), and an interaction between these factors (F(2,47)=5.908; p<0.01). Bonferroni post-hoc analysis showed that female MSEW mice have a lower 5-hydroxyindolacetic acid/5-HT ratio than female mice from SN group (p<0.01). We observed no difference in male mice (Table 6). Analysis of ***KYN*** showed no rearing group effect (F(2,47)=2.351; NS), no sex effect (F(1,47)=0.001; NS), no interaction between both factors (F(2,47)=3.069; NS). Two-way ANOVA of ***TRP/KYN*** indicated no rearing group effect (F(2,47)=1.158; NS), no sex effect (F(1,47)=1.887; NS), no interaction between these factors (F(2,47)=3.110; NS). Analysis of ***kynurenic acid*** levels showed a significant rearing group effect (F(2,47)=7.906; p<0.01), but no sex effect (F(1,47)=0.680; NS), and no interaction between these two factors (F(2,47)=2.355; NS). Bonferroni post-hoc test showed that male MSEW mice had lower kynurenic acid levels than male mice from SN group (p<0.01) (Table 6). Two-way ANOVA of the ***kynurenic acid/KYN*** ratio showed a significant rearing group effect (F(2,47)=10.266; p<0.01), but no sex effect (F(1,47)=3.265; NS) and no interaction between these factors (F(2,47)=0.277; NS). Post-hoc analysis showed that MSEW mice

had a lower kynurenic acid/KYN ratio than SN group, in male mice ($p<0.01$) and female mice ($p<0.01$) (Table 6). We observed no differences between rearing groups in ***3-hydroxykynurenine*** levels (No rearing group effect, ($F(2,47)=0.930$; NS), no sex effect ($F(1,47)=7.988$; NS), no interaction between both factors ($F2,47)=1.180$; NS)) and in the ***3-hydroxykynurenine/KYN*** ratio (No rearing group effect, ($F(2,47)=0.557$; NS), sex effect ($F(1,47)=9.555$; $p<0.01$), no interaction between these factors ($F(2,47)=0.362$; NS)). Analysis of the ***3-hydroxykynurenine/kynurenic acid ratio*** showed a significant rearing group ($F(2,47)=14.625$; $p<0.01$), sex effect ($F(2,47)=15.396$; $p<0.01$), and interaction between these two factors ($F(2,47)=3.601$; $p<0.05$). Post-hoc test showed that MSEW mice had a higher 3-hydroxykynurenine/kynurenic acid ratio than SN mice in male and female ($p<0.01$ and $p<0.05$, respectively) (Table 6). In females, one-way ANOVA of ***xanthurenic acid*** showed no rearing group effect.

The statistical analysis of this metabolic pathway in the ***HC*** showed no rearing group effect, neither sex effect, nor interaction between both factors (Table 7).

These results reflected an imbalance in the TRP-KYN pathway in MSEW mice in the PFC, suggesting that this pathway is involved in behavioural alterations induced by early life experiences whereas no differences were found between CN and SN group. The unexpected lack of significant changes found in HC may be probably attributed to the neurological heterogeneity of this brain area, as previously reported (Laugeray et al. 2010).

Table 6. Effects of rearing conditions on metabolites of the tryptophan-kynurenine pathway in the Prefrontal Cortex.

	SN		MSEW			CN	
	Male	**Female**	**Male**	**Female**		**Male**	**Female**
TRP	438,6±28,6	472,2±36,6	431,2±17,2	590,4±119,4		415,8±30,4	532,8±64,8
5-HT	61,61±6,15	51,32±6,05	75,84±4,28	65,13±8,10		72,84±3,40	51,18±5,43
5-HT/TRP	2,70±0,30	2,14±0,15	3,53±0,18	2,43±0,32		3,56±0,33	1,95±0,14
5-HIAA	15,34±1,49	29,33±3,24	17,44±1,13	25,84±2,88		17,65±1,53	37,31±3,55
5-HIAA/5-HT	5,25±0,59	12,22±1,33	4,70±0,44	6,32±1,20	**	5,98±0,37	13,44±1,61
KYN	6,03±0,60	4,71±0,69	5,13±0,59	6,94±0,88		4,88±0,67	4,33±0,45
KYN/TRP	0,28±0,03	0,20±0,02	0,23±0,02	0,26±0,04		0,23±0,02	0,17±0,02
KA	0,45±0,05	0,41±0,06	0,18±0,03	** 0,32±0,04		0,34±0,05	0,33±0,03
KA/KYN	1,47±0,12	1,87±0,34	0,71±0,09	** 1,01±0,08	**	1,46±0,16	1,59±0,14
3-HK	0,80±0,11	0,46±0,09	0,71±0,10	0,60±0,03		0,61±0,06	0,49±0,06
3-HK/KYN	2,75±0,40	1,90±0,11	2,78±0,31	1,72±0,12		2,83±0,45	2,32±0,31
3-HK/KA	37,40±5,97	25,62±4,63	73,32±8,44	** 40,40±4,07	*	34,81±5,12	29,29±3,16
XA	nd	1,20±0,25	nd	0,86±0,22		nd	1,75±0,40
XA/KYN	nd	3,32±0,55	nd	3,58±0,62		nd	7,68±1,12

Table 6. Effects of early life experiences on tryptophan-kynurenine pathway metabolites in the Prefrontal Cortex. At PD30 TRP-KYN pathway metabolites were evaluated in male and female offspring. Data are expressed as mean ± SEM of the pg/mg. N=6-8 mice per group. * $p<0.05$; ** $p<0.01$ vs. SN. Tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), no detection (nd).

Table 7. Effects of rearing conditions on metabolites of the tryptophan-kynurenine pathway in the Hippocampus.

	SN		MSEW		CN	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
TRP	676,2±43,6	420,8±41,1	780,7±25,8	445,1±60,4	659,1±28,9	396,6±34,1
5-HT	69,21±4,85	57,62±8,33	64,17±3,14	72,25±13,9	71,94±3,39	61,01±7,72
5-HT/TRP	2,07±0,04	2,77±0,37	1,68±0,08	3,20±0,56	2,36±0,11	3,04±0,22
5-HIAA	27,95±1,71	35,47±2,94	37,86±2,68	36,27±6,70	31,04±2,62	45,34±3,11
5-HIAA/5-HT	8,47±1,02	12,61±1,67	11,9±0,82	14,58±3,85	8,68±0,73	16,95±3,54
KYN	6,90±0,64	4,48±0,47	5,64±0,40	5,38±0,39	6,79±0,76	6,05±0,12
KYN/TRP	0,21±0,02	0,22±0,02	0,16±0,01	0,30±0,04	0,22±0,03	0,32±0,07
KA	0,58±0,05	0,28±0,04	0,56±0,06	0,23±0,02	0,61±0,08	0,30±0,04
KA/KYN	1,75±0,19	1,11±0,14	1,45±0,28	0,86±0,11	1,61±0,16	1,11±0,16
3-HK	0,59±0,06	0,41±0,08	0,63±0,10	0,58±0,01	0,60±0,07	0,56±0,08
3-HK/KYN	1,62±0,18	2,03±0,49	1,78±0,16	2,422±0,50	1,65±0,10	2,04±0,30
3-HK/KA	21,70±2,76	30,82±6,49	31,48±3,08	37,37±5,40	21,9±2,73	37,54±3,32
XA	nd	nd	nd	nd	nd	nd
XA/KYN	nd	nd	nd	nd	nd	nd

Table 7. Effects of early life experiences on tryptophan-kynurenine pathway metabolites in the Hippocampus. At PD30 TRP-KYN pathway metabolites were evaluated in male and female offspring. Data are expressed as mean ± SEM of the pg/mg. N=6-8 mice per group. Tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), no detection (nd).

Our results leaded us to propose that early life detrimental events, such as maternal neglect, recapitulated most of the behavioural alterations of depressive-like state in mice, showing a long-lasting affectation that persisted until adulthood and a higher sensitivity to adverse conditions in females. Our study also supported that the imbalance of the TRP-KYN metabolic pathway and the associated neuroinflammatory reactions were neurobiological alterations underlying the emotional impairments observed. Moreover, under our experimental conditions, we could not confirm the protective role of CN because of the results obtained in the behavioural test and neurochemical biomarkers assessed. Therefore, we decided to study the effect of maternal separation on addictive behaviour induced by cocaine and we focused on the effects observed in adolescent male mice in order to simplify the experimental design. Hence, in this part of the study, the CN rearing conditions were not included in the study since not appreciable differences were revealed comparing with SN conditions in the previous characterization of the behavioural model.

6. Influence of maternal separation on rewarding and addictive properties of cocaine

6.1. Evaluation of rewarding properties of cocaine in the conditioned place preference paradigm

The effect of maternal separation on the rewarding properties of cocaine (1.5, 3, 15 and 25 mg/kg, i.p.) was evaluated in adolescent male mice using the CPP paradigm. Two-way ANOVA showed a treatment effect ($F(4,101)=7.995$; $p<0,01$) without rearing group effect ($F(1,101)=0.530$; NS), with interaction between both factors ($F(4,101)=2.506$; $p<0.05$). In SN group, Bonferroni post-hoc test showed a significant effect for cocaine at doses of 3, 15 and 25 mg/kg versus saline group ($p<0.01$). Post-hoc

analysis in MSEW group indicated a significant effect for cocaine at the dose of 25 mg/kg ($p<0.01$) (Fig. 28). In addition, significant differences were observed for the dose of 25 mg/kg of cocaine when compared SN and MSEW ($p<0.05$). These results revealed that mice from MSEW group required higher doses of cocaine to display CPP.

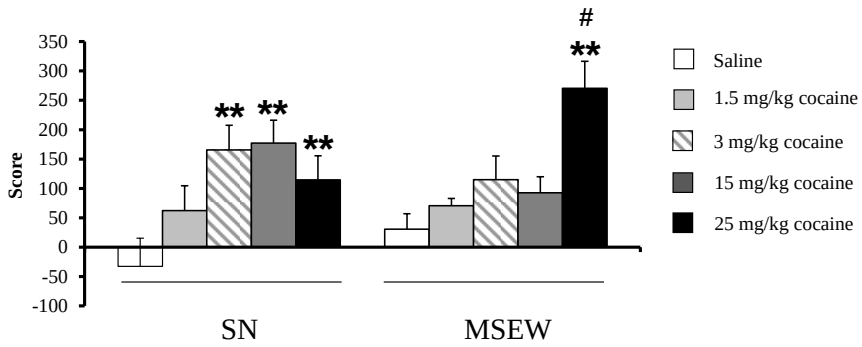


Figure 28. Effects of maternal separation on the rewarding properties of cocaine (1.5, 3, 15 and 25 mg/kg) in the conditioned place preference paradigm. Data are expressed as the mean (± SEM) of the score calculated in the CPP (see Methodology Section for details). N=8–15 mice per group. **p<0.01 vs. saline group. # p<0.05 Rearing group effect.

6.2. Expression levels of DAT and D2R proteins and DA turnover in the NAc

The effects of maternal separation on DAT, D2R and DA turnover were evaluated after performing cocaine-induced CPP. Two-way ANOVA analysis for ***DAT protein levels in the NAc*** showed a significant effect of group ($F(1,25)=5.061$; $p<0.05$), treatment ($F(2,25)=4.157$; $p<0.05$), and an interaction between these factors ($F(2,25)=5.026$; $p<0.05$). Bonferroni post-hoc analysis revealed that cocaine-treatments (3 or 15 mg/kg) significantly ($p<0.05$, $p<0.01$, respectively) increased the levels of DAT in MSEW mice when compared with the animals treated with saline (Fig. 29A). Two-way ANOVA for ***D2R protein levels*** also showed no rearing

group effect ($F(1,26)=2.37$; NS), no treatment effect ($F(2,26)=1.393$; NS) but a significant interaction between group and treatment ($F(2,25)=6.502$; $p<0.05$). Bonferroni post-hoc analysis showed that MSEW group treated with saline or cocaine (3 mg/kg) displayed a decrease in the D2R levels ($p<0.05$) when compared with the SN saline-treated group. Moreover, D2R was increased in the MSEW group treated with higher dose of cocaine (15 mg/kg) when compared with saline group ($p<0.01$). Cocaine (15 mg/kg) treatment also induced increase levels of D2R in MSEW animals when compared with the SN group treated with the same doses ($p<0.05$) (Fig. 29B).

To evaluate whether the activity of midbrain dopaminergic neurons was altered in SN and MSEW mice, after cocaine injection, DA content (data not shown), DOPAC production (data not shown) and DA turnover (as estimated by the ratio DOPAC/DA) were calculated in the NAc. Two-way ANOVA of ***DA turnover*** showed a group effect ($F(1,24)=9.53$; $p<0.01$) without treatment effect ($F(2,24)=2.65$; NS), nor interaction between these two factors ($F(2,24)=3.36$; NS). Bonferroni post-hoc test showed a significant increase in DA turnover in the MSEW group treated with higher dose of cocaine when compared with animals treated with saline ($p<0.05$). In addition, DA turnover was increased in the MSEW group treated with cocaine (15 mg/kg) when compared with SN group treated with the same dose of cocaine ($p<0.01$) (Fig. 29C).

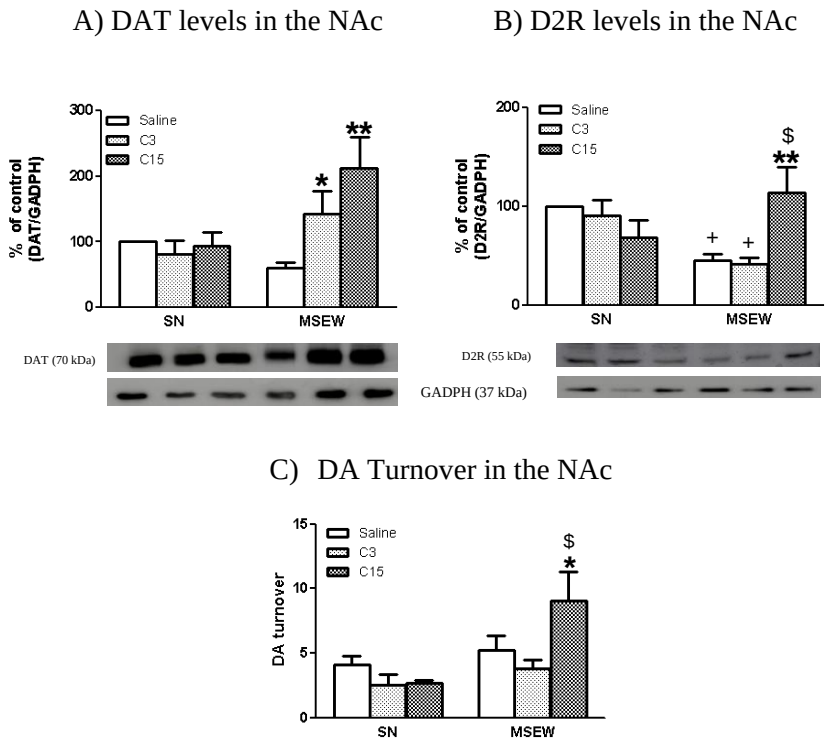


Figure 29. Effect of maternal separation on DAT, D2R levels and DA turnover in the NAc. (A, B) Densitometric analysis of specific integrated optical density (% of control) signals normalized to the corresponding GADPH levels and representative Western-blot analysis of DAT and D2R in the NAc micropunches, respectively. (C) DA turnover (as determined by the DOPAC/DA ratio) in the NAc. Each bar corresponds to mean ± SEM, N=4-6 mice per group. * $p<0.05$, ** $p<0.01$ vs. MSEW group treated with saline; + $p<0.05$ vs. SN injected with saline; & $p<0.05$ vs. SN treated with cocaine (3 mg/kg); $ $p<0.05$ vs. SN group treated with cocaine (15 mg/kg).

6.3. Expression levels of Nurr1 and Pitx3 proteins in the VTA

We also evaluated the expression levels of dopaminergic factors Nurr1 and Pitx3 in the VTA of mice previously exposed to the cocaine-induced CPP. Two-ANOVA for ***Nurr1 levels in the VTA*** showed no rearing group effect ($F(1,20)=0.64$; NS), significant effect of treatment ($F(2,20)=4.74$; $p<0.05$) and a significant interaction between both factors ($F(2,20)=7.09$; $p<0.01$). Bonferroni post-hoc test revealed a significant decrease in Nurr1 levels after cocaine-treatments (3 or 15 mg/kg; $p<0.05$; $p<0.01$, respectively) in the MSEW group when compared with saline-injected mice. Unexpectedly, MSEW animals receiving saline showed significant ($p<0.05$) elevation in Nurr1 levels versus the SN group (Fig.30A)

Two-way ANOVA for ***Pitx3 levels*** revealed rearing group effect ($F(1,20)=8.90$; $p<0.05$), no effect of treatment ($F(2,20)=2.76$; NS) with interaction between these two factors ($F(2,20)=3.92$; $p<0.05$). Bonferroni post-hoc analysis showed a significant ($p<0.05$) decreased in MSEW group treated with cocaine (15 mg/kg) when compared with the same group injected with saline. However, lower doses of cocaine (3 mg/kg) produced a significant increase ($p<0.05$) in Pitx3 levels in MSEW group versus SN group (Fig. 30B).

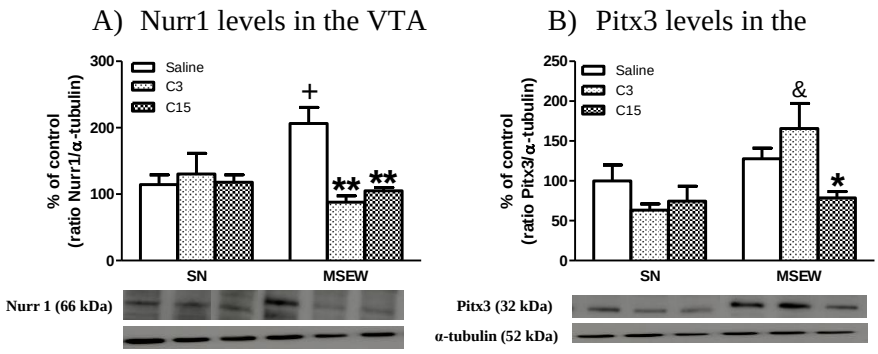


Figure 30. Effects of maternal separation on Nurr1 and Pitx3 levels in the VTA. Densitometric analysis of specific integrated optical density (% of control) signals normalized to the corresponding α-tubulin levels and representative Western-blot analysis of Nurr1 (A) and Pitx3 (B) levels in the VTA michopunches. Each bar corresponds to mean ± SEM, N=4-5 mice per group. * $p<0.05$, ** $p<0.01$ vs. MSEW group treated with saline; + $p<0.05$ vs. SN treated with saline; & $p<0.05$ vs. SN treated with cocaine (3 mg/kg).

6.4. Cocaine-induced locomotor sensitization

The effect of maternal separation on locomotor sensitization induced by cocaine (7.5 mg/kg) was evaluated in male adolescent mice. No differences in the basal locomotor activity were found between rearing groups (data not shown). The acute effect of cocaine was evaluated on day 3, and the effect of repeated cocaine treatment was evaluated after 5 days of cocaine treatment, on day 7. The possible effect of cocaine-conditioned locomotion during the repeated exposure to the locomotor activity boxes was evaluated after a single injection of saline, on day 13. Finally, the cocaine-induced sensitization to hyperlocomotor effects of cocaine was assessed after receiving a cocaine challenge injection (7.5 mg/kg, i.p.) on day 14. Three-way ANOVA with repeated measures (day factor) calculated for the locomotor activity induce by cocaine showed an effect of the rearing group, the treatment and day effect with interaction between

rearing group and day, treatment and day, and between the three factors. Values for the three-way ANOVA are shown in Table 8.

Table 8. Three-way ANOVA calculated to evaluate the effect of maternal separation on cocaine-induced locomotor sensitization. Rearing group (R), Treatment (T), Day (D).

	Cocaine-induced locomotor sensitization	
	F	**P <**
R	F(1,44)=4,722	0,05
T	F(1,44)=57,53	0,01
D	F(3,132)=116,8	0.01
R X T	F(1,44)=0,872	NS
R X D	F(3,132)=3,354	0.05
T X D	F(3,132)=8,521	0.01
R X T X D	F(3,132)=4,340	0,01

Bonferroni post-hoc test showed that acute (day 3) and repeated (day 7) administration of cocaine (7.5 mg/kg) increased the locomotor activity in both SN and MSEW groups of mice respectively when compared with the saline control group ($p<0.01$).

Repeated cocaine treatment induced significant hyperlocomotor effects in mice on day 7 (sensitization to locomotor effects) only in SN group when compared with the acute treatment ($p<0.01$). Therefore, no differences were observed between acute cocaine (day 3) and repeated cocaine effects (day 7) in MSEW group, revealing that sensitization was not properly developed in mice exposed to MSEW. Hence, the hyperlocomotion induced by repeated cocaine treatment on day 7 was attenuated in mice from MSEW comparing with mice from SN group ($p<0.01$). After five days without cocaine treatment, on day 13, mice were evaluated for the possible conditioned hyperlocomotion developed after the repeated exposure to the locomotor activity boxes during this procedure. Thus, all

groups of mice received a single injection of saline and were evaluated for the locomotor effects during 30 min. Mice pre-exposed to cocaine exhibited a significant hyperlocomotor effects comparing with mice pre-exposed to saline ($p<0.01$) on day 13, independently from the rearing conditions. On day 14, all mice received a challenge of cocaine (7.5 mg/kg) and were again evaluated in the locomotor activity boxes for locomotor sensitization to cocaine. Locomotor sensitization to cocaine was developed for SN group, revealing by a significant increase of locomotor effects on days 7 and 14 comparing with acute cocaine effects observed on day 3 ($p<0.01$). Moreover, significant differences were also observed between cocaine-induced hyperlocomotion on day 7 and on day 14 ($p<0.01$). However, mice exposed to MSEW exhibited a reduce effect of cocaine in locomotion. Hence, this group of mice exhibited increased in locomotion after cocaine challenge (day 14) when compared with effects observed in the same group of mice on day 3 ($p<0.01$) and when compared with effect of repeated treatment observed on day 7 ($p<0.01$). Nevertheless, a significant attenuation of the sensitization was observed when compared with those elicited by SN group of mice on day 14 ($p<0.01$), revealing a less strong locomotor sensitization in mice exposed to MSEW (Fig. 31).

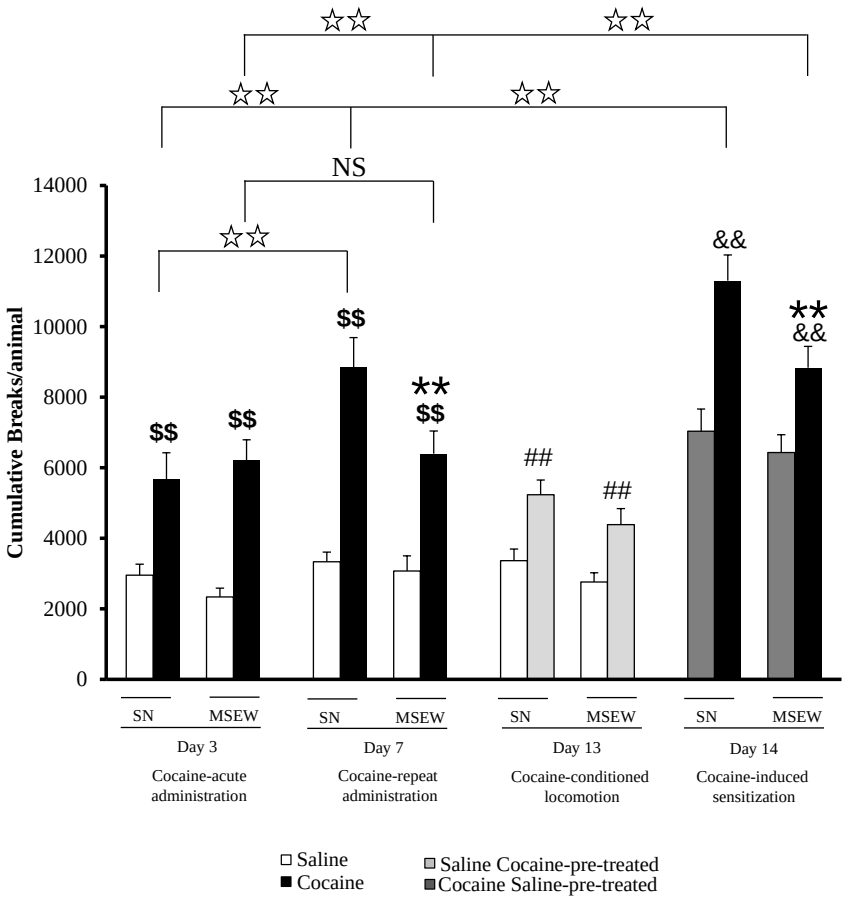


Figure 31. Effects of maternal separation on the cocaine locomotor sensitization. Data are expressed as the mean (± SEM) of cumulative breaks/animal during the first 15 min in locomotor activity boxes. N=12 mice per group. $$ $p<0.01$ treatment effect vs. saline on the same days. ## $p<0.01$, pre-treated with cocaine vs. pre-treated with saline when conditioned hyperlocomotion was evaluated on day 13. && $p<0.01$ pre-treated with cocaine vs. pre-treated with saline when cocaine-induced sensitization was evaluated. ** $p<0.01$ rearing group effect. Two white starts $p<0.01$, day effect as indicated by the arrows.

6.5. Reinforcing effects of cocaine in the self-administration paradigm

The effects of maternal separation in adolescent mice on the reinforcing properties of cocaine were evaluated using the self-administration procedure. SN and MSEW mice were trained to self-administer cocaine at doses of 1 mg/kg per infusion during 10 days. Accordingly to previous established criteria (see Methodology Section for details), the percentage of mice that achieved the acquisition criteria was 33% for SN group and 31% for MSEW group. Three-way repeated measures (day factor) ANOVA indicated hole effect and interaction between hole and day (Table 9):

Table 9. Three-way ANOVA calculated for evaluating the effect of maternal separation on cocaine-self-administration. Rearing group (R), Hole (H), Day (D).

	Cocaine self-administration	
	F	**P<**
R	F(1,17)=4,722	NS
H	F(1,17)=57,53	0,01
D	F(9,153)=116,8	NS
R X H	F(1,17)=0,872	NS
R X D	F(9,153)=3,354	NS
H X D	F(9,153)=8,521	0.01
R X H X D	F(9,153)=4,340	NS

Bonferroni post-hoc analysis revealed than SN group discriminated between the active and inactive hole from the third session (days 3 and 4, $p<0.05$; days 5 to 10, $p<0.01$), whereas MSEW group discriminated from the second session (days 2 to 10, $p<0.01$). Our results showed no significant differences between groups in cocaine self-administration (Fig. 32).

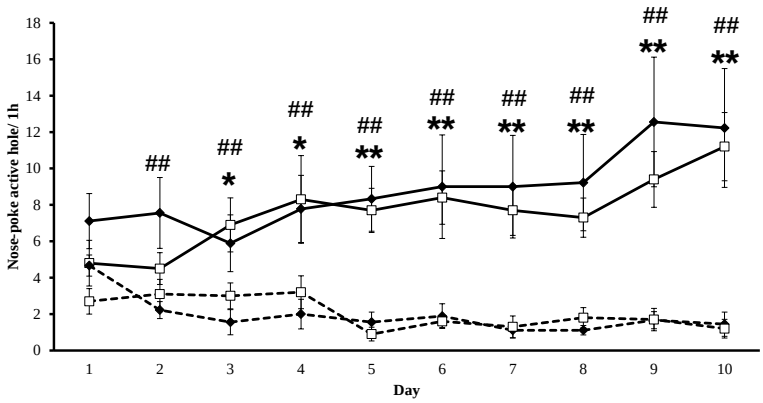


Figure 32. Effects of maternal separation on the reinforcing properties of cocaine (1mg/kg per infusion) in the self-administration procedure. Data are expressed as the mean (± SEM) of nose-poke in the active/inactive hole in the training session along the 10 days (1 h). N=9-10 mice per group. White symbols with continued line represent nose-pokes in the active hole in SN group whereas dotted line represents nose-pokes in the inactive hole. Black symbols with continued line represents nose-pokes in the active hole in MSEW group, dotted line represents nose-pokes in the inactive hole. * $p<0.05$; ** $p<0.01$ nose-pokes in the active hole vs. inactive hole in SN group. ## $p<0.01$ nose-pokes in the active hole vs. inactive hole in MSEW group.

DISCUSSION

Depression is the most prevalent psychiatric disorder in the world and one of the most important contributors to global burden of disease, causing relevant public-health problems (Murray and Lopez 2013). In humans, early life detrimental events, such as maternal neglect or abuse during childhood, represent vulnerability environmental factors to develop emotional disorders such as depression (Heim and Nemeroff 2001; Gross and Hen 2004; Heim and Binder 2012). Different behavioural models of early life stress in rodents, such as maternal separation, have been studied to elucidate the neurobiological basis of emotional and motivational alterations (George et al., 2010; Martini and Valverde, 2012; Fuentes et al., 2014). Furthermore, social environmental enrichment has emerged as a protective factor that diminishes the vulnerability of individuals to suffer psychiatric disorders (Ros-Simó and Valverde 2012). Continuing with this idea, the CN model (Branchi and Alleva 2006) provides social enrichment, thus approximating the natural ecological conditions of mice (Branchi et al. 2006a), conferring resilience against despair-like behaviour (D'Andrea et al. 2010). Therefore, the study of the behavioural and neurochemical consequences of these models is necessary in order to propose new targets to approach more effective therapeutic interventions for depression.

Moreover, early-life stress enhances the vulnerability of developing substance use disorders (Dube et al. 2003; Gerra et al. 2009), principally during adolescence since the brain is under maturation (Powell 2006; Crews et al. 2007; Giedd 2008). Growing evidence in humans indicates that depressive states are associated with substance use disorders (Cheetham et al. 2010; Rappeneau et al. 2015). Consequently, depressive patients could consume drugs to alleviate their sadness (Khantzian EJ 1985; Weiss et al. 2009).

Hence, the consequences of early-life stress during childhood may produce severe alterations during adolescence that could be maintained through adulthood. Taking this into account, *this thesis has been focused on evaluating the short and long-term consequences of early life neglect and social enrichment on emotional alterations. The influence of early-life stress on drug cocaine addiction behaviour is also investigated in this study.*

1. The evaluation of early experiences using animal models

To clarify the involvement of early life experiences in ulterior periods of lifespan such as adolescence and adulthood, *this thesis focuses in studying the detrimental or beneficial early life experiences on emotional reactivity, neurochemical consequences and drug addictive behaviour in mice*. Based in our previous experience in animal models evaluating early life experiences, we decided to carry out a maternal separation protocol due to the remarkable results previously published by our group (Martini and Valverde 2011).

Previous studies stated a compensatory maternal care after maternal separation that could counteract the deleterious effects of neglect on behaviour (Macrì et al. 2008). Hence, we chose a recent model named *"maternal separation with early weaning"* (George et al. 2010) since previous studies showed that early weaning alone without maternal separation also induces behavioural and neurochemical alterations in rodents (Kikusui and Mori 2009; Kikusui et al. 2009). In these studies, mice were weaned at PD14, and showed higher levels of anxiety and aggressive behaviour as well as neurochemical alterations at adulthood. Regarding the different animal models involving resilience to develop emotional disturbances in section *2.1.2. Positive early experiences:*

Experimental studies, the CN seems to be an appropriate model to be evaluated concomitantly with the MSEW since both could produce modifications during the postnatal period. At this point, it is important to note that the day of weaning was different in the three models previously developed, and consequently we cannot distinguish the particular contribution of the rearing condition from the early weaning in our experimental conditions.

2. Early life experiences on emotional behaviour, inflammatory response and tryptophan-kynurenine pathway.

The first objective of this study was to evaluate the behavioural and neurochemical consequences of early life neglect (MSEW) in comparison with a social enrichment model (CN) proposed as protective since this last model was reported to induce resilience to psychopathologies caused by stressful experiences (Branchi et al. 2013b). Furthermore, both models were compared with the standard laboratory rearing conditions (SN). Animals exposed to MSEW model exhibited an increase of the maternal care, presumably to compensate the exposure to maternal separation. Nevertheless, the enhancement of maternal care in mice exposed to MSEW failed to rescue neurobiological functions and behaviours impaired in the offspring as revealed by locomotion alterations, anxiety-like responses, despair-like behaviour, anhedonia and deficits in emotional memory tasks. Strikingly, these changes were revealed during adolescence and persisted into adulthood. In addition, several of these alterations were sex-dependent since a higher vulnerability in female to repeated stress exposure was observed accordingly with previous studies (Becker et al. 2007; Fuentes et al. 2014). Unexpectedly, under our experimental conditions, we cannot confirm the protective role of CN due to the

behavioural and neurochemical alterations found in the offspring. The reasons of these results will be discussed in detail later in this section.

As exposed previously, early life experiences have significant influences in shaping the normal brain development, especially in the early postnatal stages (Greenough et al. 1987; Finlay and Darlington 1995; Johnson 2001; Kolb and Gibb 2011; Cai et al. 2015). During this early stage, maternal care is needed for the normal development of emotional and cognitive behaviours of the offspring (Kaffman and Meaney 2007). Therefore, it seems relevant to evaluate maternal behaviour in experiments involving early life stress. MSEW dams displayed a higher maternal care, characterized by an increased score of arched-back posture and blanket nursing. Dams also stayed less time out of nest compared with SN and CN dams respectively. Our findings agree well with previous studies showing that early life stress in rodents, increased the active maternal behaviour (Zimmerberg and Farley 1993; Pryce et al. 2001; Own and Patel 2013; Fuentes et al. 2014). Interestingly, no differences were observed in maternal care exhibited by SN and CN groups respectively. In fact, Branchi and colleagues (2006a) showed an increase in maternal care in CN dams when they gave birth in the same day (synchronous) (Branchi et al. 2006a), whereas when dams gave birth in different days (asynchronous), the maternal care depended on the birth order of pups. Moreover in these asynchronous studies, which is our case, they did not find differences between SN and CN when compared with the older pups, although an increase in the maternal care was found in SN when compared with middle and young CN pups (Branchi et al. 2013a). In addition, a previous study (Heiderstadt et al. 2014), only distinguished between the birth order of pups when the discrepancy was of 2-7 days, but not when the discrepancy was of 1-4 days. Thus, following these criteria, we did not differentiate between the birth order of pups as they were born

with a maximum difference of 4 days. Furthermore, other CN study developed in rats found a decrease in the maternal care in CN model when compared with SN (Connors et al. 2015). Interestingly, this study differentiated between CN dams which shared the nest or had separated nests, although no differences in maternal care were found in this case. In our experiment all dams shared their nest but we realized that some dams of CN group moved the nest frequently. Consequently, evaluating the movement of the nest between CN trios and then make a correlation between this pattern and offspring behaviour should be taken into account in further studies.

Regarding our experiments, maternal care was evaluated during 16 consecutive days and statistical differences between groups started at PD5, when the maternal separation lasted longer (a total period of 8 h). Remarkably, the enhanced maternal behaviour in MSEW group was more intense at the post-separation periods, and decreased just before separation when dams and pups had remained longer together, as reported previously (Pryce et al. 2001; Llorente-Berzal et al. 2011). In addition, dams from MSEW rearing conditions progressively increased the care along the days due to the development of a sensitized behaviour associated to the repeated separations (Own and Patel 2013). Previous studies have proposed that the enhanced maternal care after early life stress periods could counteract the deleterious effects of neglect on behaviour (Macrì et al. 2008). In fact, the consequences of early stress exposure in adulthood depend on the severity of neonatal stress, being the moderate stress during neonatal periods associated with reduced stress reactivity in adulthood (Macrì et al. 2011), due to the development of capabilities to cope with stress. In contrast, our findings reveal that the negative effects of the early weaning induced by maternal separation may be stronger than those of moderate stress. Hence, our data demonstrate that maternal separation

induces strong and prolonged consequences on behaviour that could not be compensated by the enhancement of maternal postnatal care, in accordance with previous findings (Lupien et al. 2009; Fuentes et al. 2014).

The behavioural alterations found in our conditions are unlikely to be caused by nutritional changes since the differences in body weight observed during lactation (PD10 and PD17) disappeared various days after weaning (PD30) and did not re-emerge during adolescence or adulthood. Furthermore, the differences in body weight were opposite between MSEW and CN. Similar procedures to MSEW showed inconsistent results associated with the impact of maternal separation procedure in the offspring's body weight. Therefore, whereas George et al. (2010) did not find differences in the body weight of mice, other studies (McIntosh et al. 1999; Fabricius and Wo 2008) explained a decrease in the offspring´s body weight after maternal separation that was later recovered at PD65 (Fabricius and Wo 2008) or that persisted until adulthood (McIntosh et al. 1999). In these different models of maternal separation no special chow were availed in mice´s home cage, in contrast to our case, where wet chow and hydrogel were given to pups. Changes in nutritional state could be a condition needed to be taken into account since previous studies have reported the incidence of anatomical and functional alterations in the CNS in animals exposed to MSEW. These alterations include smaller brain size and developmental deficits, although no differences in body weight were reported (Duque et al. 2012; Carlyle et al. 2012). Consequently, early life stress experiences represent a vulnerability factor that induce alterations on brain development, principally during early postnatal stages (Greenough et al. 1987; Finlay and Darlington 1995; Johnson 2001; Kolb and Gibb 2011; Cai et al. 2015), modifying normal development of neural circuits controlling emotional and cognitive

behaviours in adult mammals (Kaffman and Meaney 2007). On the contrary, CN model is associated with an increase in body weight during lactation that persists until weaning and adulthood (Sayler and Salmon 1969; D'Amato et al. 2011) whereas in other studies the increase in body weight did not last into adulthood (Heiderstadt and Blizard 2011; Heiderstadt et al. 2014). In our study, these differences were only maintained during lactation and disappeared when pups were weighed few days after weaning (PD30) since our body weight differences were of less magnitude as those found in previous studies. Moreover, these discrepancies may be due to the strain mice used since the experiments before mentioned used inbred mice or BALB/c mice.

Maternal separation is related to enhanced anxiety-like behaviour in the offspring (van Oers et al. 1997; Huot et al. 2001; Martini and Valverde 2011). This goes in agreement with our results showing that adolescent mice (male and female) showed greater anxiety responses that were maintained into adulthood in females. These results highlight the long-lasting emotional effects of maternal separation and the higher sensitivity of female mice to become anxious in response to stress (Rhodes and Rubin 1999). Regarding the CN model, the results obtained in various studies were not robust. In this term, studies developed in outbred mice have shown that adult mice reared in CN displayed increased anxiety in the EPM and increased thigmotaxis in the open-field test (Branchi et al. 2006b; Branchi et al. 2006a). However, no differences in anxiety-behaviour have been found in studies using inbred mice at adulthood (Sayler and Salmon 1969; D'Andrea et al. 2007; Gracceva et al. 2009). It is important to note that these studies used adult male mice, whereas in our study we evaluated male and female at both adolescence and adulthood. Therefore, our results showed that female mice reared under CN conditions displayed an increase in anxiety-like behaviour at

adolescence that persisted into adulthood suggesting that female mice were more sensitive to this rearing condition. These results were not in agreement with a previous study in which no differences in anxiety-like behaviour in adult female mice were found (D'Andrea et al. 2010). However, the experimental conditions in the previous study were different to our experiments since they assessed the EPM in mice of 5 months old and during the dark period whereas we performed the EPM in mice of 90 days old and during the light period of the cycle. Therefore, the presentation of high aversive situations may be helpful to discriminate low from high anxiety behaviour. Thus, our experimental conditions seemed to produce higher anxiety levels than those observed in the study by D´Andrea and coworkers.

Furthermore, our results proposed that male and female MSEW mice displayed emotional alterations associated with anhedonic-like state and despair behaviour that were maintained into adulthood. Adolescent male MSEW mice showed a hypolocomotor phenotype that is likely related to apathetic behaviour (Sobin and Sackeim 1997; Lavretsky et al. 1999). This hypolocomotor phenotype did not persist into adulthood. Indeed, the hypolocomotor phenotype was only found in adolescent male mice when the spontaneous locomotor activity was assessed (Fig. 20), but not in other paradigms that indirectly provide data of locomotion (EPM and habituation for the evaluation of the nociceptive threshold). Therefore, the hypolocomotor phenotype in MSEW adolescent mice seemed not to influence the behavioural findings. In addition, our experiments displayed that adolescent MSEW group of mice had a reduction in the preference of saccharin over water as well as a decrease in the consumption of saccharin, indicating that early life stress provoked behavioural alterations related to anhedonia, a main symptom of depressive disorders present also in mice exposed to chronic stress (D'Aquila et al. 1997; Harkin et al.

2002; Strekalova et al. 2004), such as the MSEW procedure. Our results were in agreement with previous studies showing reduced saccharin consumption in two accepted models inducing depressive-like symptoms in rodents as chronic mild stress (Harkin et al. 2002; Pijlman et al. 2003; Schweizer et al. 2009), and social defeat (Furay et al. 2011; Shimamoto et al. 2015). In agreement with our results, various studies in rats using maternal separation model did not find differences in sucrose preference at adulthood (Shalev and Kafkafi 2002; Matthews and Robbins 2003). Concerning the CN, our results showed increased despair-like behaviour in adolescent male mice and adult female mice as well as a reduction in the third day of saccharin consumption in adolescent male mice. Previous results showed an increase in the floating time in the forced swimming test in adult male and female mice in the CN, although they interpreted this immobility as successful and adaptive strategy to preserve energy (Branchi et al. 2010; D'Andrea et al. 2010) since the administration of antidepressants reduced the floating time in CN mice, whereas the floating time in SN did not change. Moreover, they found an increase in the sucrose preference test in adult female from CN group at basal conditions (D'Andrea et al. 2010) that persisted after isolation, whereas adult male were more sensitive displaying a reduction in the sucrose preference after isolation (D'Andrea et al. 2010). In our study, we did not find differences between SN and CN group excepting the third day of saccharin consumption in adolescent male mice suggesting that under our experimental conditions the CN is not as protective as reported by Branchi and colleagues.

As expected, a significant sex effect was found in various behavioural tests in our study, since female mice from MSEW group were more sensitive to adverse emotional conditions than males exposed to the same experimental conditions. These results were consistent with experimental

(Palanza 2001; Palanza et al. 2001; Renoir et al. 2011) and clinical studies (Kessler 2003; Altemus 2006) which exposed a higher prevalence for depressive symptoms in females. Hence, women could be more sensitive than men to early negative experiences, showing a higher vulnerability to develop pathopsychological traits after early life detrimental events (Weissman et al. 1996; Kudielka and Kirschbaum 2005; Sherin and Nemeroff 2011). Concerning this issue, gonadal hormones display profound effects in the CNS. In this sense, estradiol and progesterone have a role in the anxiety-like behaviour in rodents through their action on estrogenous receptors (Lund et al. 2005; Osterlund et al. 2005; Tomihara et al. 2009; Spiteri et al. 2010). Therefore, experimental studies showed that estradiol and progesterone display anxiolytic effects under different procedures to evaluate anxiety in rodents (Zimmerberg and Farley 1993; Mora et al. 1996; Fernández-Guasti and Picazo 1997; Palanza et al. 2001). Furthermore, we have also validated that low progesterone and estrogenous levels such as seen in diestrus and in male mice can enhance susceptibility to anxiogenic effects induced by cocaine (Martini et al. 2014). Various studies also revealed the effects of the estrous cycle in the responsiveness of stress (Zimmerberg and Farley 1993; Mora et al. 1996; Fernández-Guasti and Picazo 1997; Palanza et al. 2001). These results indicate a decrease in behavioural levels of anxiety during proestrus phase, when levels of ovarian steroids hormones are higher (Butcher et al. 1974). In our study, the estrous cycle was not assessed because of the complexity of the experimental procedure and the number of different manipulations achieved in the mice during the complete procedure. Consequently, we cannot discard the involvement of hormonal milieu in the emotional alterations observed in female mice after the exposure to MSEW or CN. Indeed, a recent study has shown that maternal separation impaired the influence of estrous cycle on feeding behaviour in rats (Iwasaki and Inoue 2015).

A substantial proportion of depressive patients also exhibit cognitive deficits and retardation, affecting in particular to several memory functions, including declarative memory, executive functions and emotional processing (Eriksson et al. 2012). Several studies have associated cognitive impairments found in depressive patients with monoaminergic neurotransmission alterations (Tsuji et al. 2003; Clark et al. 2009). Indeed, acute TRP depletion modulates 5-HT availability in the brain and seems to play an important role in mediating resistance to distracting negative information (Roiser et al. 2008). In line with these findings, our results also revealed that MSEW female mice displayed emotional memory impairments, as shown in clinical studies (Roiser et al. 2008) and different animal models (Eriksson et al. 2012), leading us to propose that the emotional memory impairments observed in MSEW females, in the passive avoidance task, could be related to alterations in the TRP-KYN pathway. In our experimental conditions, the response observed in female MSEW mice could not be related to alterations in locomotor activity or in the electric nociceptive threshold. Actually, previous experiments have found a reduction in thermal pain sensitivity after maternal separation (Weaver et al. 2007), whereas our experiments showed no changes in the electric nociceptive threshold neither for rearing conditions nor for sex (Table 5). Moreover, alterations in the performance in passive avoidance test could be attributed to neuroinflammatory reactions induced by the chronic exposure to MSEW (Elmore et al. 2015; Lykhmus et al. 2015). Interestingly, adolescent female CN mice displayed a slight reduction in the latency to enter the black compartment related to emotional memory alterations at adolescence without statistical significance, whereas they showed impaired emotional memory at adulthood. It is important to note that female CN mice also had an increase in the neuroinflammatory response at adolescence. However, no alterations in memory and learning have been described before in CN

model (D'Andrea et al. 2007; Heiderstadt et al. 2014), although the performed tests were the Morris water maze (D'Andrea et al. 2007) and the Lashley III maze (Heiderstadt et al. 2014) which involved spatial memory dependent on the hippocampal formation (Vorhees and Williams 2006; Lashley 1963) whereas the passive avoidance task involves the amygdala (LeDoux 1993; Phelps 2004). Hence, the different brain areas involved may underlie different memory processes. In addition, no differences in pain threshold have been described in CN model.

Recent preclinical and clinical findings propose the implication of the immune system in depressive disorders through a mechanism involving inflammatory responses (Motivala et al. 2005; Miller et al. 2009; Felger and Lotrich 2013). To explore this possibility, we evaluated peripheral and central inflammatory responses related to microglial activation, a primary source of pro-inflammatory cytokines in the brain, in males and females exposed to different rearing conditions. Our experiments revealed that inflammatory reactions were developed in discrete brain areas, such as PFC and hippocampal areas (CA1, CA3 and DG). Thus, we observed significant microglial activation in female mice exposed to MSEW and CN, whereas no changes were observed in males exposed to the same conditions. The highest levels of activated microglial cells were found in the PFC and CA1, and CA3 hippocampal areas, brain structures involved in the control of emotional and cognitive functions (Palazidou 2012). Moreover, hippocampal areas participate in the regulation of the activity of HPA axis, also involved in the pathophysiology of depression as explain before (Nestler 2014; Cai et al. 2015). In fact, microglial cells activation was higher in CA1 and CA3 hippocampal regions in female from MSEW and CN groups, being these regions susceptible to be damaged after the excessive activation of the HPA axis by stress, and also critically involved in emotional behaviours (Fanselow 2000). Our data

agreed well with previous studies showing a higher activation of microglia in depressive mice exposed to chronic unpredictable mild stress (Farooq et al. 2012). In contrast, astrocytic activation showed no significant differences in the number of immunoreactive cells between groups. However, post-mortem analysis in brain tissues from patients suffering depression have shown a decrease in the number of astrocytes in brain areas related to emotional control (Miguel-Hidalgo et al. 2000; Rajkowska and Miguel-Hidalgo 2007), although opposite data was also reported by different authors (Davis et al. 2002; Malchow et al. 2014). These last studies did not reveal changes in the number or density of astrocytes in post-mortem brain tissues from patients with a diagnostic of depression. Therefore, evidences pointed out that stress regulates astrocytic proliferation (Sharma et al. 1992; Lambert et al. 2000), whereas others studies reported diminished GFAP-immunoreactive astrocytes in neocortex and HC (Czéh et al. 2006; Banasr et al. 2010). These contradictory results could be due to different methodological factors including stress induction, temporal schedules, species and strain, and require further investigations to standardize experimental conditions in order to be adequately considered.

At peripheral level, cytokine IL-6 levels did not reveal significant differences between groups, despite that a tendency to increase cytokine levels was observed in female MSEW group, accordingly with our behavioural and neuroinflammatory results. However, an increase in pro-inflammatory cytokines serum levels was found in maternal separated rats after forced swimming test (Réus et al. 2015b) suggesting that stress induces the activation of the immune system after early adverse experiences. Furthermore, several studies using alternative models to induce depression (such as social defeat or learned helplessness) showed increased IL-6 levels in serum (Sukoff Rizzo et al. 2012; Hodes et al.

2014), whereas another study revealed no changes in IL-6 in serum from depressive mice (Farooq et al. 2012). Clinical studies in humans reported that IL-6 production appeared increased in adolescents with antecedents of childhood adversity as a predictive biomarker of depression (Miller and Cole 2012). Taken together, the apparent discrepancy between basic and clinical researches could be attributed to the time point in which the samples were collected. In our case serum was collected at PD30 between 9-13 days after weaning, thus allowing time for the development of compensatory mechanisms.

Interestingly, the different impact of MSEW on microglial and astrocytic cell proliferation could play a relevant role in the development of depressive states (Miller et al. 2009), and could be attributed to an unbalance in the TRP-KYN metabolism pathway (Miller et al. 2009; Myint 2012). Alterations in this metabolic pathway have been formerly associated with impairments in the functionality of different neurotransmitter systems including those related to the control of mood (Miller et al. 2009). Therefore, KYN is preferentially converted to kynurenic acid in astrocytes and in quinolenic acid in microglial cells. A neuroprotective role has been attributed to kynurenic acid in the brain whereas quinolenic acid seems to have a neurotoxic activity (Borland and Michael 2004; Müller and Schwarz 2007; McNally et al. 2008). This process emphasizes the deleterious effects of inflammatory reactions in neuronal repair and survival. In this context, previous studies developed in our laboratory showed that a single episode of maternal separation induced a decrease in BDNF levels in discrete brain areas including HC and amygdala (Martini and Valverde 2011), demonstrating the association between neuroplasticity and early life negative experiences. In consequence, female MSEW group exhibited decreased 5-HT metabolism in the PFC, revealed by a decrease in the 5-hydroxyindoleacetic acid and

5-hydroxyindoleacetic acid/5-HT ratio, leading us to propose a functional reduction of 5-HT neurotransmission that may be involved in the development of a depressive-like state in animals exposed to MSEW. We could consider that these functional modifications in 5-HT neurotransmission are not exclusively due to the availability of the 5-HT, and alterations in the functionality of different components of the signaling neurotransmitter pathways could also contribute to the alteration observed. These possibilities were not addressed in the present study and further experiments would be required to clarify these points. In accordance, reduced expression of 5-HT1A receptors was proposed to explain the anxiety-like behaviour displayed in rats exposed to prenatal stress (White and Birkle 2001) or maternal separation (Leventopoulos et al. 2009; Bravo et al. 2014). Furthermore, post-synaptic 5-HT1A receptors are located mainly in the PFC (Palacios et al. 1990), and the reduction of 5-HT transporter sites and 5-HT1A receptors levels has been observed in this brain area in post-mortem patients of depression (López-Figueroa et al. 2004), suggesting that cortical 5-HT neurotransmission is altered (Le François et al. 2015), as proposed in our present findings. In addition, mice lacking CB1 cannabinoid receptors, a proposed animal model of depression (Valverde and Torrens 2012) displayed alterations in 5-HT1A and 5-HT2C receptors within brain areas related to the control of emotional responses (Aso et al. 2008). Analysis of the TRP-KYN pathway in MSEW mice revealed a decrease in the relation between kynurenic acid and KYN, showing a decrease in the production of kynurenic acid, a metabolite with neuroprotective effects (Borland and Michael 2004; Sas et al. 2007; Klein et al. 2013). Interestingly, the ratio between 3-hydroxykynurenine and kynurenic acid, the neurotoxic and the neuroprotective metabolites respectively resulted in a significant increase for mice reared in MSEW conditions. Consequently, we propose that the KYN metabolic pathway could be unbalanced towards the production of

neurotoxic metabolites such as 3-hydroxykynurenine and quinolenic acid. Meanwhile, no changes were found in 3-hydroxykynurenine levels, we propose that KYN is metabolized to quinolenic acid through anthranilic acid, an alternative metabolic pathway (See Fig. 2). Nevertheless, the concentration of quinolenic acid from our selected brain areas was too small to be accurately quantified. In agreement, clinical studies have stated an imbalance between neuroprotective and neurotoxic metabolites of KYN metabolic pathway measured in serum samples from depressive patients (Myint et al. 2007) showing lower KYN/kynurenic acid ratio in depressive patients that correlates with a decrease in the neuroprotective metabolites activity. Strikingly, this imbalance in the KYN pathway was not reversed after chronic antidepressant treatment (Heyes et al. 1992). In addition, supplementary clinical studies supported our hypothesis and showed that quinolenic acid in post-mortem brain tissues (PFC and HC) was increased in patients with depression and bipolar disorder, respectively (Steiner et al. 2011). Furthermore, an increase of KYN and 3-hydroxyanthranilic/KYN ratio in serum was also observed in adolescents with melancholic depression (Gabbay et al. 2010), supporting our idea that the KYN could be metabolized to quinolenic acid via anthranilic acid pathway. The unexpected lack of significant changes found in HC may be probably attributed to the neurological heterogeneity of this brain area, as previously reported (Laugeray et al. 2010). Taken together, our results demonstrated for the first time that mice from MSEW displayed an unbalance in the KYN metabolic pathway that could explain the impaired emotional behaviour observed in these animals, and led us to propose the involvement of these metabolic pathways in the pathophysiology of depressive states.

Under our experimental conditions, we could not confirm the protective role of CN, probably because this breeding condition involves energetic

cost and increases the competition for food in pups (Hayes 2000). Moreover, it is important to note that in the CN cages increased pup numbers causes a reduction in the space per pup that could involve overcrowding as well as increased humidity or ammonia levels that could induce detrimental health effects (Heiderstadt et al. 2014). Indeed, in our experimental conditions we had to change the CN cage more frequently than SN or MSEW cages. In addition, as a result of previous studies (Goody and Kitchen 2001), it has been reported that delayed weaning facilitates depressive-like behaviour in rodents (Farshim et al., 2011) due to a mechanism involving opioid neurotransmission (Filliol et al. 2000; Goody and Kitchen 2001). In fact, delayed weaning seemed to specifically activate delta-opiod receptors, and consequently produced an unbalance in mu-opioid receptor activity, as reported (Filliol et al. 2000). Delayed weaning altered delta-mu opioid function that was associated with the longer consumption of maternal milk (Goody and Kitchen 2001). Consequently, the increased pup number and the prolonged lactation in the CN group could contribute to explain the unexpected emotional behavioural alterations observed in CN group in our experimental conditions. Therefore, our results did not support a beneficial role in offspring reared in CN for all stated above, thus we decided not to evaluate the CN group in drug additive behaviour.

3. Maternal separation influences cocaine-addictive behaviour through the modulation of the dopaminergic system.

The impairment of brain reward function is hypothesized to be involved in the symptoms of depression, reducing positive affect and inducing anhedonia (Nestler et al. 2002; Nestler and Carlezon 2006). This impairment may also be related to the high comorbidity between depression and substance use disorders (Merikangas et al. 1999; Cheetham et al. 2010). In addition, maltreatment early in life induces pathological disturbances leading to negative emotional states (Koob 2008; Lupien 2014; Van Dam et al. 2014). The individuals suffering early maltreatment are prone to consume drugs as a results of negative reinforcement mechanisms, which could results in increased reward thresholds (Koob 2008; Koob 2009b). Therefore, *the second objective of the study was to evaluate the influence of negative early life experiences induced by MSEW on the rewarding and reinforcing properties of cocaine after an acute or repeated treatment. Aberrant plasticity on dopaminergic reward circuits was also investigated in adolescent male mice*. It is important to note that whereas research on drugs of abuse in animal models has been increased in the past decade, experiments exploring drug abuse, adolescence, *and stress* have received poor attention (Burke and Miczek 2014).

The mechanisms involved in psychiatric comorbidity may differ depending on the temporal course of its development (i.e. depression followed by drug abuse or vice versa) (Volkow 2004). Therefore, in our experimental conditions, MSEW seems to be an appropriate experimental model for studying the implication of depression on the substance use disorders since as stated in the previous section, MSEW induces behavioural impairments and neurochemical alterations that recapitulate

depressive symptoms in rodents. Consequently, we evaluated the drug addictive behaviour during adolescence in mice exposed to MSEW since little is known about the effects of negative early experiment at this age. In fact, adolescence is an important period of life in which brain regions involved in emotional control and impulsivity are under maturation processes (Spear 2000; Crews et al. 2007; Giedd 2008).

Our results in the cocaine-induced conditioned place preference paradigm indicated that mice from SN group conditioned at different doses (3 mg/kg, 15 mg/kg and 25 mg/kg), whereas MSEW mice conditioned only at the highest dose of cocaine used (25 mg/kg), suggesting that MSEW mice needed higher doses of cocaine to experience the rewarding effects of the psychostimulant.

One possibility to explain these results could be attributed to possible cognitive deficits induced by MSEW in mice since several studies have reported learning and memory deficits in rodents exposed to maternal separation (Wang et al. 2011; Thomas et al. 2015). The decrease of the rewarding effects of cocaine in CPP was not due to cognitive impairments since MSEW mice were able to respond at the highest dose of cocaine (25 mg/kg), indicating that mice appropriately responded to the contextual cues. Thus, we hypothesized that MSEW induced modifications in the dopaminergic system altering the rewarding properties of cocaine. Consequently, our results are in agreement with the anhedonia displayed by MSEW mice at adolescence in the saccharin test (Gracia-Rubio et al. 2015). In addition, previous studies have shown decreased rewarding effects of amphetamine (Papp et al. 1991) and morphine (Valverde et al. 1997) in animals exposed to chronic mild stress. Moreover, in a neonatal model of stress has also been reported a decrease in the rewarding properties of cocaine evaluated in the conditioned place preference (Hays

et al. 2012). However, a different model of maternal separation showed an enhancement in rewarding effects of morphine evaluated in the conditioned place preference (Vazquez et al. 2007), as well as in adolescent social defeat mice using cocaine (Rodríguez-Arias et al. 2015), suggesting an increase sensitivity in stressed mice.

Strikingly, acute administration of cocaine (7.5 mg/kg) induced similar enhanced locomotor activity in mice exposed to SN and MSEW groups. Although the repeated administration of cocaine increased locomotor activity in SN and MSEW groups of mice, this increase was higher in SN group, indicating reduced cocaine effect in MSEW mice. No differences were found between groups when saline was administered after the repeated treatment of cocaine to evaluate the cocaine-conditioned locomotion, although mice pretreated with cocaine displayed higher locomotion activity than saline-pretreated mice. In addition, cocaine-pretreated SN mice showed higher locomotor activity than MSEW, whereas no differences were found between saline-pretreated groups. We concluded that MSEW was involved in the capability of cocaine to induce behavioural sensitization. Particularly, MSEW decreases the intensity of behavioural sensitization compared with SN group probably reducing the ability of cocaine to induce the neuroadaptations for the development of this behaviour. In addition, it is important to note that the dose of cocaine selected for our study (7.5 mg/kg) was lower than those used in previous studies evaluating behaviour sensitization (Kikusui et al. 2005). Therefore, our findings evaluating sensitization in mice exposed to MSEW are in line with our results observed in the place preference paradigm that is a reduction in the cocaine effects in mice exposed to MSEW. Consequently, the lower sensitivity to the psychostimulant might lead to increase experiences with the drug to experience its pleasurable

effects which is strongly linked to the initial phases for the development of addictive disorders (Planeta and Marin 2002).

Previous studies using maternal separation models showed no consistent results regarding the psychostimulants effects. Thus, an increase in cocaine sensitization after repeated maternal separation was observed in adult mice (Matthews et al. 1999; Kosten et al. 2003; Kikusui et al. 2005). However, other studies have shown a less robust behavioural response after cocaine administration in female adult rats after maternal separation (Li et al. 2003). Moreover, no differences in behavioural sensitization in adolescent male rats after maternal separation were reported (Planeta and Marin 2002). Our experimental conditions were different to those reported in the previous studies for both, the maternal separation and the sensitization procedures, and consequently, clear conclusions are difficult to be drawn.

We have also evaluated cocaine-induced reinforcing effects in the i.v. self-administration procedure using an operant paradigm. In the present study, no differences between rearing conditions were found in the self-administration procedure probably due to the dose of cocaine (1 mg/kg per infusion) selected and the duration of the training period. Therefore, further studies are required to complete this particular study.

In this sense, preclinical studies have found that negative early life experiences can modulate the rewarding mesolimbic circuits in adulthood; both increasing reward induced behavioural-reinforcement (Matthews et al. 1999; Kosten et al. 2003) or reducing rewarding responses, probably due to the development of anhedonia (Martini and Valverde 2011; O'Connor et al. 2015). Other studies evaluating different models of repeated stress such as social defeat have also shown controversial data. Consequently, whereas adult social defeat stress resulted in an escalated

cocaine self-administration in socially stressed adult mice (Han et al. 2015), no differences were found in social stressed adolescent mice (Rodríguez-Arias et al. 2015).

Regarding the molecular evaluation of the dopaminergic system in mice exposed to MSEW, a decrease in D2R protein expression levels in the NAc and an increase of the transcriptional factors Nurr1 in the VTA during adolescence were found. However, no differences in DAT, DA turnover or the transcriptional factor Pitx3 were observed. The decrease in D2R in saline-treated MSEW mice could reflect the anhedonia-like effects observed in behavioural studies, and thus the attenuation of rewarding activity of mesolimbic system. Accordingly, a model of PTSD also displayed a decrease in D2R in NAc and showed anhedonia-like behaviour as well as a decrease in cocaine intake in self-administration procedure (Enman et al. 2015). In agreement, mice exposed to stressful situations during several days also showed a D2R density reduction in the NAc (Puglisi-Allegra et al. 1991). At this point, we must consider the hypothesis by Volkow and colleagues, proposing that high levels of D2R confer resilience to developed substance use disorders whereas low levels of D2R increase vulnerability to suffer this psychiatric disorder (Volkow et al. 2002). This hypothesis supports our results regarding the lack of reward observed in conditioned place preference, since MSEW mice probably required high doses of cocaine to experience reward. Accordingly, the administration of D2R antagonist, sulpiride (100 mg/kg, i.p.), reduced cocaine sensitization (Mattingly et al., 1994) and blocked cocaine, morphine and alcohol rewarding effects in the place preference paradigm (Matsuzawa et al. 1999; Rezayof et al. 2002; Nazarian et al. 2004). Interestingly, knockout mice lacking D2R receptors exhibited a higher cocaine self-administration than wild-type littermates only at higher doses of cocaine, whereas no differences were found between

genotypes when using lower doses of cocaine (0.03 and 3.2 mg/kg per infusion) (Caine et al. 2002). These results support that D2 receptors seem not to be essentials in the manifestations of reinforcing effects of cocaine, and their participation in reward responses depends of the range of cocaine doses assayed. Other studies have also evaluated the participation of maternal separation on the dopaminergic function, reporting no differences in D2R density in adult mice (Vazquez et al. 2007) while a model of prenatal stress found an increase in the expression of these dopaminergic receptors (Katunar et al. 2009). These results are in line with cocaine sensitization results here reported. Therefore, the decrease of D2 receptors could be related to the reduction of cocaine-induced locomotion in mice exposed to MSEW. In addition, our data also show the existence of a compensatory mechanism to enhance the impaired dopaminergic activity through an increased expression of Nurr1 protein levels in the VTA. Nurr1 is a transcription factor essential for the differentiation of midbrain DA neurons (Jankovic et al. 2005). Therefore, D2R KO mice exhibited an enhancement of Nurr1 levels in the VTA and substance nigra whereas no differences were found in other brain regions such as cortex or habenular nuclei (Tseng et al. 2000). Moreover, both 6-hydroxydopamine lesions in the striatum (Ojeda et al. 2003) and the exposure to prenatal stress (Katunar et al. 2009) produced a rapid increase of Nurr1 expression in the substance nigra, as a compensatory mechanism to the dopaminergic activity lost.

Our results in SN mice during cocaine-induced conditioned place preference showed no changes in protein expression of DAT, D2R, Nurr1, and Pitx3 as well as in DA turnover, suggesting that cocaine did not produce neuroadaptations in the dopaminergic system after a sub-chronic cocaine treatment. It is important to note that mice were sacrificed 48 h after the last cocaine injection when no expected residual psychostimulant

effects remained. In this sense, decreases in Nurr1 and Pitx3 protein were found after 14 days of cocaine treatment, without changes after acute or shorter cocaine treatment. Surprisingly, no differences were observed in TH, DAT, VMAT2 or D2R after 14 days of cocaine treatment (Leo et al. 2007). In those conditions, the decreased expression of Nurr1 and Pitx3 can be viewed as a homeostatic response to increased DA neurotransmission after the cocaine treatment. Moreover, the lack of modulation of other genes affecting DA neurotransmission after chronic cocaine administration suggests that the partial down-regulation of Nurr1 and Pitx3 may not be enough to modify the expression of some putative downstream target genes (Leo et al. 2007). However, the morphine (acute and chronic treatments) and the morphine withdrawal enhances the expression of the former dopaminergic factors inducing alterations in the expression of DAT, VMAT and D2R (García-Pérez et al. 2014; García-Pérez et al. 2015). Our results lead us to suggest that mice exposed to MSEW developed a hypofunction of the dopaminergic system as revealed in saline-treated mice. This dopaminergic dysfunction is hypothesized to contribute to drug-seeking and drug-taking behaviours (Morgan et al. 2002; Koob and Volkow 2010). Therefore, the intermittent administration of cocaine in MSEW mice was able to induce neuroadaptations in the dopaminergic system, which would be involved in compensatory mechanisms. Consequently, MSEW exposed to cocaine displayed increased protein expression levels of DAT, D2R and DA turnover as well as decreased of Nurr1 and Pitx3 when compared with saline-treated MSEW mice. However, further experiments would be needed to clarify the implication of MSEW model in the acute and long-term regulation of the dopaminergic system. Although our results showed an attenuation of cocaine rewarding effects and cocaine-induced sensitization, we conclude that MSEW is an appropriate model to investigate the influence of anhedonia (a main symptom of depression) on substance use disorders,

due to the higher vulnerability to develop addictive disorders presumably related to the reduction in dopaminergic activity in circuits controlling reward.

CONCLUSIONS

The **final conclusions** of the present Doctoral Thesis are the following:

1. Detrimental early life events such as maternal separation with early weaning reproduce most of the behavioural impairments associated with emotional alterations in mice, including locomotor changes, anxiety-like responses, despair- and anhedonia-like behaviours. Interestingly, these changes are revealed during adolescence and persist into adulthood.

2. Maternal separation induces cognitive impairments associated with emotional learning and memory tasks. These deficits cannot be attributed to alterations in locomotor activity or in the nociceptive threshold to electric stimuli.

3. Mothers exposed to maternal separation display an increase of the maternal care behaviour, presumably to compensate the exposure to stressful situation of repeated episodes of separation. However, the enhancement of maternal care failed to recover the complete neurobiological functions and behaviours impaired in the offspring.

4. Mice exposed to maternal separation show higher microglia activation related to neuroinflammatory processes in discrete brain areas, such as PFC and hippocampal areas (CA1, CA3).

5. Mice exposed to maternal separation display an unbalance in the tryptophan-kynurenine metabolism pathway in the PFC, suggesting that this pathway is involved in the emotional alterations induced by negative early life experiences.

6. Most of the neurochemical and behavioural alterations here reported are sex-dependent since a higher vulnerability to repeated stress exposure was observed in female.

7. The protective role of communal nest cannot be confirmed because of the neurochemical and behavioural alterations found in these mice, probably due to energetic cost and competition for food in pups in these breeding conditions.

8. Mice exposed to maternal separation show attenuated rewarding properties of cocaine in accordance with the development of anhedonia as exposed above. No differences were found in self-administration of cocaine.

9. Maternal separation causes a decrease in behavioural sensitization induced by cocaine suggesting the impaired development of the neuroadaptive processes induced by the repeated treatment with cocaine.

10. Mice exposed to maternal separation showed decreased protein expression levels of D2 receptors and an increased of the transcriptional factor Nurr1, suggesting a hypofunctionality of the dopaminergic system accordingly with the anhedonia-like behaviour observed in mice exposed to maternal separation.

11. Maternal separation with early weaning could be considered as a useful model to investigate the comorbidity between depression and substance use disorder as it induces alterations in emotional and drug addictive behaviours.

REFERENCES

Aimone JB, Li Y, Lee SW, et al (2014) Regulation and Function of Adult Neurogenesis: From Genes to Cognition. Physiol Rev 94:991–1026. doi: 10.1152/physrev.00004.2014

Aisa B, Elizalde N, Tordera R, et al (2009) Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. Hippocampus 19:1222–31. doi: 10.1002/hipo.20586

Albert PR (2015) Why is depression more prevalent in women? J Psychiatry Neurosci 40:219–21.

Alcaro A, Huber R, Panksepp J (2007) Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. Brain Res Rev 56:283–321. doi: 10.1016/j.brainresrev.2007.07.014

Al-Harbi KS (2012) Treatment-resistant depression: therapeutic trends, challenges, and future directions. Patient Prefer Adherence 6:369–88. doi: 10.2147/PPA.S29716

Altemus M (2006) Sex differences in depression and anxiety disorders: potential biological determinants. Horm Behav 50:534–8. doi: 10.1016/j.yhbeh.2006.06.031

American Psychiatric Association (2013) Diagnostic and Statistical Manual of Mental Disorders.

Andersen SL (2003) Trajectories of brain development: point of vulnerability or window of opportunity? Neurosci Biobehav Rev 27:3–18.

Andersen SL, Teicher MH (2009) Desperately driven and no brakes: developmental stress exposure and subsequent risk for substance abuse. Neurosci Biobehav Rev 33:516–24. doi: 10.1016/j.neubiorev.2008.09.009

Anderson G, Berk M, Dean O, et al (2014) Role of immune-inflammatory and oxidative and nitrosative stress pathways in the etiology of depression: Therapeutic implications. CNS Drugs 28:1–10. doi: 10.1007/s40263-013-0119-1

Angold A, Rutter M (2008) Effects of age and pubertal status on depression in a large clinical sample. Dev Psychopathol 4:5. doi: 10.1017/S0954579400005538

Anthony J, Warner L, Kessler R. Comparative epidemiology of dependence on tobacco, alcohol, controlled substances, and

inhalants: Basic findings from the National

Armario A (2010) Activation of the hypothalamic–pituitary–adrenal axis by addictive drugs: different pathways, common outcome. Trends Pharmacol Sci 31:318–325. doi: 10.1016/j.tips.2010.04.005

Artigas F (2013) Serotonin receptors involved in antidepressant effects. Pharmacol Ther 137:119–31. doi: 10.1016/j.pharmthera.2012.09.006

Aso E, Ozaita A, Valdizán EM, et al (2008) BDNF impairment in the hippocampus is related to enhanced despair behavior in CB 1 knockout mice. J Neurochem 105:565–572. doi: 10.1111/j.1471-4159.2007.05149.x

Aso E, Renoir T, Mengod G, et al (2009) Lack of CB1 receptor activity impairs serotonergic negative feedback. J Neurochem 109:935–44. doi: 10.1111/j.1471-4159.2009.06025.x

Bäckman C, Perlmann T, Wallén A, et al (1999) A selective group of dopaminergic neurons express Nurr1 in the adult mouse brain. Brain Res 851:125–32.

Bale TL (2015) Epigenetic and transgenerational reprogramming of brain development. Nat Rev Neurosci 16:332–44. doi: 10.1038/nrn3818

Banasr M, Chowdhury GMI, Terwilliger R, et al (2010) Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. Mol Psychiatry 15:501–11. doi: 10.1038/mp.2008.106

Bannon MJ, Pruetz B, Manning-Bog AB, et al (2002) Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. Proc Natl Acad Sci U S A 99:6382–5. doi: 10.1073/pnas.092654299

Barr AM, Fiorino DF, Phillips AG (1999) Effects of withdrawal from an escalating dose schedule of d-amphetamine on sexual behavior in the male rat. Pharmacol Biochem Behav 64:597–604.

Barrett SP, Darredeau C, Pihl RO (2006) Patterns of simultaneous polysubstance use in drug using university students. Hum Psychopharmacol 21:255–63. doi: 10.1002/hup.766

Becker A, Grecksch G, Kraus J, et al (2002) Rewarding effects of ethanol and cocaine in mu opioid receptor-deficient mice. Naunyn Schmiedebergs Arch Pharmacol 365:296–302. doi: 10.1007/s00210-002-0533-2

Becker JB, Monteggia LM, Perrot-Sinal TS, et al (2007) Stress and disease: is being female a predisposing factor? J Neurosci 27:11851–5. doi: 10.1523/JNEUROSCI.3565-07.2007

Belin D, Balado E, Piazza PV, Deroche-Gamonet V (2009) Pattern of intake and drug craving predict the development of cocaine addiction-like behavior in rats. Biol Psychiatry 65:863–8. doi: 10.1016/j.biopsych.2008.05.031

Bello EP, Mateo Y, Gelman DM, et al (2011) Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. Nat Neurosci 14:1033–8. doi: 10.1038/nn.2862

Belmaker RH, Agam G (2008) Major depressive disorder. N Engl J Med 358:55–68. doi: 10.1056/NEJMra073096

Belsky J, Fearon RMP (2002) Early attachment security, subsequent maternal sensitivity, and later child development: does continuity in development depend upon continuity of caregiving? Attach Hum Dev 4:361–87. doi: 10.1080/14616730210167267

Belzung C, Griebel G (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 125:141–9.

Berrendero F, Kieffer BL, Maldonado R (2002) Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in mu-opioid receptor knock-out mice. J Neurosci 22:10935–40.

Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391–431. doi: 10.1007/s00213-006-0578-x

Berton O, Nestler EJ (2006) New approaches to antidepressant drug discovery: beyond monoamines. Nat Rev Neurosci 7:137–51. doi: 10.1038/nrn1846

Bhatia SK, Bhatia SC (2007) Childhood and adolescent depression. Am Fam Physician 75:73–80.

Bilici M, Efe H, Köroğlu MA, et al (2001) Antioxidative enzyme activities and lipid peroxidation in major depression: alterations by antidepressant treatments. J Affect Disord 64:43–51.

Biskup CS, Sánchez CL, Arrant A, et al (2012) Effects of acute tryptophan depletion on brain serotonin function and concentrations of dopamine and norepinephrine in C57BL/6J and BALB/cJ mice. PLoS One 7:e35916. doi: 10.1371/journal.pone.0035916

Blanco E, Bilbao A, Luque-Rojas MJ, et al (2011) Attenuation of cocaine-induced conditioned locomotion is associated with altered expression of hippocampal glutamate receptors in mice lacking LPA1 receptors. Psychopharmacology (Berl) 220:27–42. doi: 10.1007/s00213-011-2446-6

Borland LM, Michael AC (2004) Voltammetric study of the control of striatal dopamine release by glutamate. J Neurochem 91:220–9. doi: 10.1111/j.1471-4159.2004.02708.x

Branchi I, Alleva E (2006) Communal nesting, an early social enrichment, increases the adult anxiety-like response and shapes the role of social context in modulating the emotional behavior. Behav Brain Res 172:299–306. doi: 10.1016/j.bbr.2006.05.019

Branchi I, Cirulli F (2014) Early experiences: Building up the tools to face the challenges of adult life. Dev Psychobiol. doi: 10.1002/dev.21235

Branchi I, Curley JP, D'Andrea I, et al (2013a) Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology 38:522–532. doi: 10.1016/j.psyneuen.2012.07.010

Branchi I, D'Andrea I, Cirulli F, et al (2010) Shaping brain development: mouse communal nesting blunts adult neuroendocrine and behavioral response to social stress and modifies chronic antidepressant treatment outcome. Psychoneuroendocrinology 35:743–51. doi: 10.1016/j.psyneuen.2009.10.016

Branchi I, D'Andrea I, Fiore M, et al (2006a) Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. Biol Psychiatry 60:690–6. doi: 10.1016/j.biopsych.2006.01.005

Branchi I, D'Andrea I, Sietzema J, et al (2006b) Early social enrichment augments adult hippocampal BDNF levels and survival of BrdU-positive cells while increasing anxiety- and "depression"-like behavior. J Neurosci Res 83:965–73. doi: 10.1002/jnr.20789

Branchi I, Santarelli S, D'Andrea I, Alleva E (2013b) Not all stressors are equal: early social enrichment favors resilience to social but not physical stress in male mice. Horm Behav 63:503–9. doi: 10.1016/j.yhbeh.2013.01.003

Bravo JA, Dinan TG, Cryan JF (2014) Early-life stress induces persistent

alterations in 5-HT1A receptor and serotonin transporter mRNA expression in the adult rat brain. Front Mol Neurosci 7:24. doi: 10.3389/fnmol.2014.00024

Bredy TW, Zhang TY, Grant RJ, et al (2004) Peripubertal environmental enrichment reverses the effects of maternal care on hippocampal development and glutamate receptor subunit expression. Eur J Neurosci 20:1355–62. doi: 10.1111/j.1460-9568.2004.03599.x

Brown ES, Varghese FP, McEwen BS (2004) Association of depression with medical illness: does cortisol play a role? Biol Psychiatry 55:1–9.

Brummelte S, Galea LAM (2010) Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. Horm Behav 58:769–79. doi: 10.1016/j.yhbeh.2010.07.012

Brunson KL, Kramár E, Lin B, et al (2005) Mechanisms of late-onset cognitive decline after early-life stress. J Neurosci 25:9328–38. doi: 10.1523/JNEUROSCI.2281-05.2005

Brydon L, Harrison N a., Walker C, et al (2008) Peripheral Inflammation is Associated with Altered Substantia Nigra Activity and Psychomotor Slowing in Humans. Biol Psychiatry 63:1022–1029. doi: 10.1016/j.biopsych.2007.12.007

Burke AR, Miczek KA (2014) Stress in adolescence and drugs of abuse in rodent models: role of dopamine, CRF, and HPA axis. Psychopharmacology (Berl) 231:1557–80. doi: 10.1007/s00213-013-3369-1

Burton GJ, Jauniaux E (2011) Oxidative stress. Best Pract Res Clin Obstet Gynaecol 25:287–99. doi: 10.1016/j.bpobgyn.2010.10.016

Buss RR, Sun W, Oppenheim RW (2006) Adaptive roles of programmed cell death during nervous system development. Annu Rev Neurosci 29:1–35. doi: 10.1146/annurev.neuro.29.051605.112800

Butcher RL, Collins WE, Fugo NW (1974) Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 94:1704–8. doi: 10.1210/endo-94-6-1704

Cabib S, Puglisi-Allegra S (2012) The mesoaccumbens dopamine in coping with stress. Neurosci Biobehav Rev 36:79–89. doi:

10.1016/j.neubiorev.2011.04.012

Cadoret RJ, O'Gorman TW, Heywood E, Troughton E (1985) Genetic and environmental factors in major depression. J Affect Disord 9:155–164. doi: 10.1016/0165-0327(85)90095-3

Cai N, Chang S, Li Y, et al (2015) Molecular signatures of major depression. Curr Biol 25:1146–56. doi: 10.1016/j.cub.2015.03.008

Caine SB, Negus SS, Mello NK, et al (2002) Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. J Neurosci 22:2977–88. doi: 20026264

Calabrese F, Rossetti AC, Racagni G, et al (2014) Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity. Front Cell Neurosci 8:430. doi: 10.3389/fncel.2014.00430

Cameron DL, Williams JT (1993) Dopamine D1 receptors facilitate transmitter release. Nature 366:344–7. doi: 10.1038/366344a0

Carlyle BC, Duque A, Kitchen RR, et al (2012) Maternal separation with early weaning: a rodent model providing novel insights into neglect associated developmental deficits. Dev Psychopathol 24:1401–16. doi: 10.1017/S095457941200079X

Carpenter GL, Stacks AM (2009) Developmental effects of exposure to Intimate Partner Violence in early childhood: A review of the literature. Child Youth Serv Rev 31:831–839. doi: 10.1016/j.childyouth.2009.03.005

Carroll BJ, Cassidy F, Naftolowitz D, et al (2007) Pathophysiology of hypercortisolism in depression. Acta Psychiatr Scand Suppl 90–103. doi: 10.1111/j.1600-0447.2007.00967.x

Casey BJ, Jones RM, Hare TA (2008) The adolescent brain. Ann N Y Acad Sci 1124:111–26. doi: 10.1196/annals.1440.010

Cass WA, Gerhardt GA, Gillespie K, et al (1993) Reduced Clearance of Exogenous Dopamine in Rat Nucleus Accumbens, but Not in Dorsal Striatum, Following Cocaine Challenge in Rats Withdrawn from Repeated Cocaine Administration. J Neurochem 61:273–283. doi: 10.1111/j.1471-4159.1993.tb03565.x

Castrén E, Rantamäki T (2010) The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of

developmental plasticity. Dev Neurobiol 70:289–97. doi: 10.1002/dneu.20758

Castrén E, Võikar V, Rantamäki T (2007) Role of neurotrophic factors in depression. Curr Opin Pharmacol 7:18–21. doi: 10.1016/j.coph.2006.08.009

Cattaneo A, Macchi F, Plazzotta G, et al (2015) Inflammation and neuronal plasticity: a link between childhood trauma and depression pathogenesis. Front Cell Neurosci 9:40. doi: 10.3389/fncel.2015.00040

Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10:295–304. doi: 10.1038/nrg2540

Champagne DL, Bagot RC, van Hasselt F, et al (2008) Maternal care and hippocampal plasticity: evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. J Neurosci 28:6037–45. doi: 10.1523/JNEUROSCI.0526-08.2008

Chaney A, Carballedo A, Amico F, et al (2014) Effect of childhood maltreatment on brain structure in adult patients with major depressive disorder and healthy participants. J Psychiatry Neurosci 39:50–9. doi: 10.1503/jpn.120208

Cheetham A, Allen NB, Yücel M, Lubman DI (2010) The role of affective dysregulation in drug addiction. Clin Psychol Rev 30:621–34. doi: 10.1016/j.cpr.2010.04.005

Chen BT, Bowers MS, Martin M, et al (2008) Cocaine but not natural reward self-administration nor passive cocaine infusion produces persistent LTP in the VTA. Neuron 59:288–97. doi: 10.1016/j.neuron.2008.05.024

Chopra K, Kumar B, Kuhad A (2011) Pathobiological targets of depression. Expert Opin Ther Targets 15:1–22. doi: 10.1517/14728222.2011.553603

Christmas DM, Potokar JP, Davies SJC (2011) A biological pathway linking inflammation and depression: Activation of indoleamine 2,3-dioxygenase. Neuropsychiatr Dis Treat 7:431–439. doi: 10.2147/NDT.S17573

Chrousos GP, Kino T (2007) Glucocorticoid action networks and complex

psychiatric and/or somatic disorders. Stress 10:213–9. doi: 10.1080/10253890701292119

Chung S, Hedlund E, Hwang M, et al (2005) The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons. Mol Cell Neurosci 28:241–52. doi: 10.1016/j.mcn.2004.09.008

Civelli O, Bunzow JR, Grandy DK (1993) Molecular diversity of the dopamine receptors. Annu Rev Pharmacol Toxicol 33:281–307. doi: 10.1146/annurev.pa.33.040193.001433

Clark DB, Kirisci L, Tarter RE (1998) Adolescent versus adult onset and the development of substance use disorders in males. Drug Alcohol Depend 49:115–21.

Clark L, Chamberlain SR, Sahakian BJ (2009) Neurocognitive mechanisms in depression: implications for treatment. Annu Rev Neurosci 32:57–74. doi: 10.1146/annurev.neuro.31.060407.125618

Clarke AS (1993) Social rearing effects on HPA axis activity over early development and in response to stress in rhesus monkeys. Dev Psychobiol 26:433–46. doi: 10.1002/dev.420260802

Clarke H, Flint J, Attwood AS, Munafò MR (2010) Association of the 5-HTTLPR genotype and unipolar depression: a meta-analysis. Psychol Med 40:1767–78. doi: 10.1017/S0033291710000516

Clarke LE, Barres BA (2013) Emerging roles of astrocytes in neural circuit development. Nat Rev Neurosci 14:311–21. doi: 10.1038/nrn3484

Clelland CD, Choi M, Romberg C, et al (2009) A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210–3. doi: 10.1126/science.1173215

Cohen P, Cohen J, Kasen S, et al (1993) An Epidemiological Study of Disorders in Late Childhood and Adolescence?I. Age- and Gender-Specific Prevalence. J Child Psychol Psychiatry 34:851–867. doi: 10.1111/j.1469-7610.1993.tb01094.x

Connors EJ, Migliore MM, Pillsbury SL, et al (2015) Environmental enrichment models a naturalistic form of maternal separation and shapes the anxiety response patterns of offspring. Psychoneuroendocrinology 52:153–67. doi: 10.1016/j.psyneuen.2014.10.021

Conrad KL, Tseng KY, Uejima JL, et al (2008) Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature 454:118–21. doi: 10.1038/nature06995

Conway KP, Compton W, Stinson FS, Grant BF (2006) Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. J Clin Psychiatry 67:247–57.

Coppen A (1969) Defects in monoamine metabolism and their possible importance in the pathogenesis of depressive syndromes. Psychiatr Neurol Neurochir 72:173–80.

Cowan WM, Fawcett JW, O'Leary DD, Stanfield BB (1984) Regressive events in neurogenesis. Science 225:1258–65.

Crews F, He J, Hodge C (2007) Adolescent cortical development: a critical period of vulnerability for addiction. Pharmacol Biochem Behav 86:189–99. doi: 10.1016/j.pbb.2006.12.001

Cruceanu C, Alda M, Nagy C, et al (2013) H3K4 tri-methylation in synapsin genes leads to different expression patterns in bipolar disorder and major depression. Int J Neuropsychopharmacol 16:289–99. doi: 10.1017/S1461145712000363

Czéh B, Simon M, Schmelting B, et al (2006) Astroglial plasticity in the hippocampus is affected by chronic psychosocial stress and concomitant fluoxetine treatment. Neuropsychopharmacology 31:1616–26. doi: 10.1038/sj.npp.1300982

D'Amato FR, Zanettini C, Sgobio C, et al (2011) Intensification of maternal care by double-mothering boosts cognitive function and hippocampal morphology in the adult offspring. Hippocampus 21:298–308. doi: 10.1002/hipo.20750

D'Andrea I, Alleva E, Branchi I (2007) Communal nesting, an early social enrichment, affects social competences but not learning and memory abilities at adulthood. Behav Brain Res 183:60–6. doi: 10.1016/j.bbr.2007.05.029

D'Andrea I, Gracci F, Alleva E, Branchi I (2010) Early social enrichment provided by communal nest increases resilience to depression-like behavior more in female than in male mice. Behav Brain Res 215:71–6. doi: 10.1016/j.bbr.2010.06.030

D'Aquila PS, Collu M, Gessa GL, Serra G (1997) Dizocilpine prevents

the enhanced locomotor response to quinpirole induced by repeated electroconvulsive shock. Eur J Pharmacol 330:11–4.

Dahl RE (2004) Adolescent brain development: a period of vulnerabilities and opportunities. Keynote address. Ann N Y Acad Sci 1021:1–22. doi: 10.1196/annals.1308.001

Dailly E, Chenu F, Renard CE, Bourin M (2004) Dopamine, depression and antidepressants. Fundam Clin Pharmacol 18:601–607. doi: 10.1111/j.1472-8206.2004.00287.x

Danese A, Pariante CM, Caspi A, et al (2007) Childhood maltreatment predicts adult inflammation in a life-course study.

Dantzer R, O'Connor JC, Freund GG, et al (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46–56. doi: 10.1038/nrn2297

Dantzer R, O'Connor JC, Lawson M a., Kelley KW (2011) Inflammation-associated depression: From serotonin to kynurenine. Psychoneuroendocrinology 36:426–436. doi: 10.1016/j.psyneuen.2010.09.012

Davis S, Thomas A, Perry R, et al (2002) Glial fibrillary acidic protein in late life major depressive disorder: an immunocytochemical study. J Neurol Neurosurg Psychiatry 73:556–60.

de Graaf-Peters VB, Hadders-Algra M (2006) Ontogeny of the human central nervous system: what is happening when? Early Hum Dev 82:257–66. doi: 10.1016/j.earlhumdev.2005.10.013

de Quervain DJ, Roozendaal B, Nitsch RM, et al (2000) Acute cortisone administration impairs retrieval of long-term declarative memory in humans. Nat Neurosci 3:313–4. doi: 10.1038/73873

De Roo M, Klauser P, Garcia PM, et al (2008) Spine dynamics and synapse remodeling during LTP and memory processes. Prog Brain Res 169:199–207. doi: 10.1016/S0079-6123(07)00011-8

Delgado PL, Moreno FA (2000) Role of norepinephrine in depression. J Clin Psychiatry 61 Suppl 1:5–12.

Dhir A, Kulkarni SK (2011) Nitric oxide and major depression. Nitric Oxide 24:125–131. doi: 10.1016/j.niox.2011.02.002

Di Chiara G (1999) Drug addiction as dopamine-dependent associative learning disorder. Eur J Pharmacol 375:13–30. doi: 10.1016/S0014-

2999(99)00372-6

Dimitsantos E, Escorihuela RM, Fuentes S, et al (2007) Litter size affects emotionality in adult male rats. Physiol Behav 92:708–16. doi: 10.1016/j.physbeh.2007.05.066

Disse E, Bussier AL, Veyrat-Durebex C, et al (2010) Peripheral ghrelin enhances sweet taste food consumption and preference, regardless of its caloric content. Physiol Behav 101:277–281. doi: 10.1016/j.physbeh.2010.05.017

Dong Y, Nestler EJ (2014) The neural rejuvenation hypothesis of cocaine addiction. Trends Pharmacol Sci 35:374–83. doi: 10.1016/j.tips.2014.05.005

Doremus-Fitzwater TL, Spear LP (2010) Age-related differences in amphetamine sensitization: effects of prior drug or stress history on stimulant sensitization in juvenile and adult rats. Pharmacol Biochem Behav 96:198–205. doi: 10.1016/j.pbb.2010.05.005

Dröge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47–95. doi: 10.1152/physrev.00018.2001

Dube SR, Felitti VJ, Dong M, et al (2003) Childhood abuse, neglect, and household dysfunction and the risk of illicit drug use: the adverse childhood experiences study. Pediatrics 111:564–72.

Duncan GJ, Brooks-Gunn J, Klebanov PK (1994) Economic deprivation and early childhood development. Child Dev 65:296–318.

Duque A, Coman D, Carlyle BC, et al (2012) Neuroanatomical changes in a mouse model of early life neglect. Brain Struct Funct.459-72. doi: 10.1007/s00429-011-0350-9.

Duval F, Mokrani M-C, Monreal-Ortiz JA, et al (2006) Cortisol hypersecretion in unipolar major depression with melancholic and psychotic features: dopaminergic, noradrenergic and thyroid correlates. Psychoneuroendocrinology 31:876–88. doi: 10.1016/j.psyneuen.2006.04.003

Eiland L, Romeo RD (2013) Stress and the developing adolescent brain. Neuroscience 249:162–71. doi: 10.1016/j.neuroscience.2012.10.048

Elmore MRP, Lee RJ, West BL, Green KN (2015) Characterizing newly repopulated microglia in the adult mouse: impacts on animal behavior, cell morphology, and neuroinflammation. PLoS One 10:e0122912. doi: 10.1371/journal.pone.0122912

Enman NM, Arthur K, Ward SJ, et al (2015) Anhedonia, Reduced Cocaine Reward, and Dopamine Dysfunction in a Rat Model of Posttraumatic Stress Disorder. Biol Psychiatry. doi: 10.1016/j.biopsych.2015.04.024

Enthoven L, Oitzl MS, Koning N, et al (2008) Hypothalamic-pituitary-adrenal axis activity of newborn mice rapidly desensitizes to repeated maternal absence but becomes highly responsive to novelty. Endocrinology 149:6366–77. doi: 10.1210/en.2008-0238

Eriksson TM, Delagrange P, Spedding M, et al (2012) Emotional memory impairments in a genetic rat model of depression: involvement of 5-HT/MEK/Arc signaling in restoration. Mol Psychiatry 17:173–184. doi: 10.1038/mp.2010.131

Esplugues J V (2002) NO as a signalling molecule in the nervous system. Br J Pharmacol 135:1079–95. doi: 10.1038/sj.bjp.0704569

Everitt BJ, Belin D, Economidou D, et al (2008) Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. Philos Trans R Soc Lond B Biol Sci 363:3125–35. doi: 10.1098/rstb.2008.0089

Everitt BJ, Robbins TW (2015) Drug Addiction: Updating Actions to Habits to Compulsions Ten Years On. Annu Rev Psychol. doi: 10.1146/annurev-psych-122414-033457

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22:3312–20. doi: 20026356

Fabricius K, Wo ÆG (2008) The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus. 403–416. doi: 10.1007/s00429-007-0169-6

Fanselow MS (2000) Contextual fear, gestalt memories, and the hippocampus. Behav Brain Res 110:73–81.

Farooq RK, Isingrini E, Tanti A, et al (2012) Is unpredictable chronic mild stress (UCMS) a reliable model to study depression-induced neuroinflammation? Behav Brain Res 231:130–7. doi: 10.1016/j.bbr.2012.03.020

Farshim P, Bailey A, Chakrabarti B, Swann J, Saddy D, Kitchen I. (2011). Delayed weaning influences depressive, but not anxiety-like or social behaviour in rats. Comunication

Felger JC, Lotrich FE (2013) Inflammatory cytokines in depression:

Neurobiological mechanisms and therapeutic implications. Neuroscience 246:199–229. doi: 10.1016/j.neuroscience.2013.04.060

Fernández-Guasti A, Picazo O (1997) Anxiolytic actions of diazepam, but not of buspirone, are influenced by gender and the endocrine stage. Behav Brain Res 88:213–8.

Fernstrom JD, Fernstrom MH (2007) Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. J Nutr 137:1539S–1547S; discussion 1548S.

Filliol D, Ghozland S, Chluba J, et al (2000) Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. Nat Genet 25:195–200. doi: 10.1038/76061

Finlay BL, Darlington RB (1995) Linked regularities in the development and evolution of mammalian brains. Science 268:1578–84.

Fischman MW, Schuster CR, Javaid J, et al (1985) Acute tolerance development to the cardiovascular and subjective effects of cocaine. J Pharmacol Exp Ther 235:677–82.

Flint AJ, Black SE, Campbell-Taylor I, et al (1993) Abnormal speech articulation, psychomotor retardation, and subcortical dysfunction in major depression. J Psychiatr Res 27:309–19.

Flint J, Kendler KS (2014) The genetics of major depression. Neuron 81:484–503. doi: 10.1016/j.neuron.2014.01.027

Fodor A, Klausz B, Pintér O, et al (2012) Maternal neglect with reduced depressive-like behavior and blunted c-fos activation in Brattleboro mothers, the role of central vasopressin. Horm Behav 62:539–551. doi: 10.1016/j.yhbeh.2012.09.003

Fombonne E (2001) The Maudsley long-term follow-up of child and adolescent depression: 1. Psychiatric outcomes in adulthood. Br J Psychiatry 179:210–217. doi: 10.1192/bjp.179.3.210

Fox HC, Jackson ED, Sinha R (2009) Elevated cortisol and learning and memory deficits in cocaine dependent individuals: relationship to relapse outcomes. Psychoneuroendocrinology 34:1198–207. doi: 10.1016/j.psyneuen.2009.03.007

Francis PT (2003) Glutamatergic systems in Alzheimer's disease. Int J Geriatr Psychiatry 18:S15–21. doi: 10.1002/gps.934

Freud S (1984) Uber coca. J Subst Abuse Treat 1:206–217.

Fuentes S, Daviu N, Gagliano H, et al (2014) Sex-dependent effects of an early life treatment in rats that increases maternal care: vulnerability or resilience? Front Behav Neurosci 8:56. doi: 10.3389/fnbeh.2014.00056

Furay AR, McDevitt RA, Miczek KA, Neumaier JF (2011) 5-HT1B mRNA expression after chronic social stress. Behav Brain Res 224:350–7. doi: 10.1016/j.bbr.2011.06.016

Gabbay V, Klein RG, Katz Y, et al (2010) The possible role of the kynurenine pathway in adolescent depression with melancholic features. J Child Psychol Psychiatry 51:935–43. doi: 10.1111/j.1469-7610.2010.02245.x

Gabilondo A, Rojas-Farreras S, Vilagut G, et al (2010) Epidemiology of major depressive episode in a southern European country: results from the ESEMeD-Spain project. J Affect Disord 120:76–85. doi: 10.1016/j.jad.2009.04.016

García-Pérez D, López-Bellido R, Rodríguez RE, et al (2015a) Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. Brain Struct Funct 220:1901–19. doi: 10.1007/s00429-014-0761-5

García-Pérez D, López-Bellido R, Hidalgo JM, et al (2015b) Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. Addict Biol 20:104–19. doi: 10.1111/adb.12083

García-Pérez D, Núñez C, Laorden ML, Milanés MV (2014) Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. Addict Biol. doi: 10.1111/adb.12209

George ED, Bordner KA, Elwafi HM, Simen AA (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

Gerra G, Leonardi C, Cortese E, et al (2009) Childhood neglect and parental care perception in cocaine addicts: relation with psychiatric symptoms and biological correlates. Neurosci Biobehav Rev 33:601–10. doi: 10.1016/j.neubiorev.2007.08.002

Gerstein DR, Harwood HJ (1992) Repeating Cycles of Cocaine Use and Abuse.

Ghozland S, Matthes HWD, Simonin F, et al (2002) Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. J Neurosci 22:1146–54.

Giedd JN (2008) The teen brain: insights from neuroimaging. J Adolesc Health 42:335–43. doi: 10.1016/j.jadohealth.2008.01.007

Giedd JN (2004) Structural magnetic resonance imaging of the adolescent brain. Ann N Y Acad Sci 1021:77–85. doi: 10.1196/annals.1308.009

Golden SA, Christoffel DJ, Heshmati M, et al (2013) Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression. Nat Med 19:337–44. doi: 10.1038/nm.3090

Goldman-Rakic PS (1987) Development of cortical circuitry and cognitive function. Child Dev 58:601–22.

González Llona I, Tumuluru S, González-Torres MÁ, Gaviria M (2015) Cocaína: una revisión de la adicción y el tratamiento. Rev la Asoc Española Neuropsiquiatría 35:555–571. doi: 10.4321/S0211-57352015000300008

González-Sepúlveda M, Pozo OJ, Marcos J, Valverde O (2015) Chronic pain causes a persistent anxiety state leading to increased ethanol intake in CD1 mice. J Psychopharmacol. doi: 10.1177/0269881115622238

Goody RJ, Kitchen I (2001) Influence of maternal milk on functional activation of delta-opioid receptors in postnatal rats. J Pharmacol Exp Ther 296:744–748.

Gorman JM, Docherty JP (2010) A hypothesized role for dendritic remodeling in the etiology of mood and anxiety disorders. J Neuropsychiatry Clin Neurosci 22:256–64. doi: 10.1176/appi.neuropsych.22.3.256

Gould F, Clarke J, Heim C, et al (2012) The effects of child abuse and neglect on cognitive functioning in adulthood. J Psychiatr Res 46:500–506. doi: 10.1016/j.jpsychires.2012.01.005

Gourion D, Arseneault L, Vitaro F, et al (2008) Early environment and major depression in young adults: A longitudinal study. Psychiatry Res 161:170–176. doi: 10.1016/j.psychres.2007.07.026

Gracceva G, Venerosi A, Santucci D, et al (2009) Early social enrichment affects responsiveness to different social cues in female mice. Behav Brain Res 196:304–9. doi: 10.1016/j.bbr.2008.09.023

Gracia-Rubio I, Moscoso-Castro M, Pozo OJ, et al (2015) 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

Grant BF, Stinson FS, Hasin DS, et al (2005) Prevalence, correlates, and comorbidity of bipolar I disorder and axis I and II disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. J Clin Psychiatry 66:1205–15.

Graybiel AM (2000) The basal ganglia. Curr Biol 10:R509–11.

Greenough WT, Black JE, Wallace CS (1987) Experience and brain development. Child Dev 58:539–59.

Gross C, Hen R (2004) The developmental origins of anxiety. Nat Rev Neurosci 5:545–552. doi: 10.1038/nrn1429

Grueter BA, Rothwell PE, Malenka RC (2012) Integrating synaptic plasticity and striatal circuit function in addiction. Curr Opin Neurobiol 22:545–51. doi: 10.1016/j.conb.2011.09.009

Gudmundsson P, Skoog I, Waern M, et al (2007) The relationship between cerebrospinal fluid biomarkers and depression in elderly women. Am J Geriatr Psychiatry 15:832–8. doi: 10.1097/JGP.0b013e3180547091

Guiard BP, El Mansari M, Merali Z, Blier P (2008) Functional interactions between dopamine, serotonin and norepinephrine neurons: an in-vivo electrophysiological study in rats with monoaminergic lesions. Int J Neuropsychopharmacol 11:625–39. doi: 10.1017/S1461145707008383

Guire ES, Oh MC, Soderling TR, Derkach VA (2008) Recruitment of calcium-permeable AMPA receptors during synaptic potentiation is regulated by CaM-kinase I. J Neurosci 28:6000–9. doi: 10.1523/JNEUROSCI.0384-08.2008

Hackman DA, Farah MJ, Meaney MJ (2010) Socioeconomic status and the brain: mechanistic insights from human and animal research. Nat Rev Neurosci 11:651–9. doi: 10.1038/nrn2897

Haddad LM (1978) 1978: Cocaine in perspective. JACEP 8:374–6.

Hamon M, Lanfumey L, el Mestikawy S, et al (1990) The main features of central 5-HT1 receptors. Neuropsychopharmacology 3:349–360.

Han X, Albrechet-Souza L, Doyle MR, et al (2015) Social stress and escalated drug self-administration in mice II. Cocaine and dopamine in the nucleus accumbens. Psychopharmacology (Berl) 232:1003–10. doi: 10.1007/s00213-014-3734-8

Harkin A, Houlihan DD, Kelly JP (2002) Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine. J Psychopharmacol 16:115–23.

Hasin DS, Goodwin RD, Stinson FS, Grant BF (2005) Epidemiology of major depressive disorder: results from the National Epidemiologic Survey on Alcoholism and Related Conditions. Arch Gen Psychiatry 62:1097–106. doi: 10.1001/archpsyc.62.10.1097

Hatten ME (1999) Central nervous system neuronal migration. Annu Rev Neurosci 22:511–39. doi: 10.1146/annurev.neuro.22.1.511

Hatzigiakoumis DS, Martinotti G, Giannantonio M Di, Janiri L (2011) Anhedonia and substance dependence: clinical correlates and treatment options. Front psychiatry 2:10. doi: 10.3389/fpsyt.2011.00010

Hayes L (2000) To nest communally or not to nest communally: a review of rodent communal nesting and nursing. Anim Behav 59:677–688. doi: 10.1006/anbe.1999.1390

Hays SL, McPherson RJ, Juul SE, et al (2012) Long-term effects of neonatal stress on adult conditioned place preference (CPP) and hippocampal neurogenesis. Behav Brain Res 227:7–11. doi: 10.1016/j.bbr.2011.10.033

Heiderstadt KM, Blizard DA (2011) Increased juvenile and adult body weights in BALB/cByJ mice reared in a communal nest. J Am Assoc Lab Anim Sci 50:484–7.

Heiderstadt KM, Vandenbergh DJ, Gyekis JP, Blizard DA (2014) Communal nesting increases pup growth but has limited effects on adult behavior and neurophysiology in inbred mice. J Am Assoc Lab Anim Sci 53:152–60.

Heim C, Binder EB (2012) Current research trends in early life stress and depression: Review of human studies on sensitive periods, gene-environment interactions, and epigenetics. Exp Neurol 233:102–111.

doi: 10.1016/j.expneurol.2011.10.032

Heim C, Nemeroff C (2001) The Role of Childhood Trauma in the Neurobiology of Mood and Anxiety Disorders: Preclinical and Clinical Studies. Biol Psychiatry 49:1023–1039.

Heim C, Newport DJ, Mletzko T, et al (2008) The link between childhood trauma and depression: insights from HPA axis studies in humans. Psychoneuroendocrinology 33:693–710. doi: 10.1016/j.psyneuen.2008.03.008

Heim C, Shugart M, Craighead WE, Nemeroff CB (2010) Neurobiological and psychiatric consequences of child abuse and neglect. Dev Psychobiol 52:671–690. doi: 10.1002/dev.20494

Herman JP, Figueiredo H, Mueller NK, et al (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24:151–80.

Herman JP, Ostrander MM, Mueller NK, Figueiredo H (2005) Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. Prog Neuropsychopharmacol Biol Psychiatry 29:1201–13. doi: 10.1016/j.pnpbp.2005.08.006

Herrero Álvarez S. Cocaína en el derecho penal español. Adicciones 2001;13(supl 2): 227-247

Heyes MP, Saito K, Crowley JS, et al (1992) Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. Brain 115 (Pt 5:1249–73.

Hindorff LA, Sethupathy P, Junkins HA, et al (2009) Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc Natl Acad Sci U S A 106:9362–7. doi: 10.1073/pnas.0903103106

Hodes GE, Pfau ML, Leboeuf M, et al (2014) Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress. Proc Natl Acad Sci 111:16136–16141. doi: 10.1073/pnas.1415191111

Huot RL, Thrivikraman K V, Meaney MJ, Plotsky PM (2001) Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Psychopharmacology (Berl)

158:366–73. doi: 10.1007/s002130100701

Huttenlocher PR, Dabholkar AS (1997) Regional differences in synaptogenesis in human cerebral cortex. J Comp Neurol 387:167–78.

Huttenlocher PR, De Courten C, Garey LJ, Van der Loos H (1984) Synaptic development in human cerebral cortex. Int J Neurol 16-17:144–54.

Hwang D-Y, Hong S, Jeong J-W, et al (2009) Vesicular monoamine transporter 2 and dopamine transporter are molecular targets of Pitx3 in the ventral midbrain dopamine neurons. J Neurochem 111:1202–12. doi: 10.1111/j.1471-4159.2009.06404.x

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. Annu Rev Neurosci 29:565–98. doi: 10.1146/annurev.neuro.29.051605.113009

Iwasaki S, Inoue K (2015) Maternal separation impedes the estrous cyclic changes of feeding behavior in female rats. Exp Anim. doi: 10.1538/expanim.15-0020

Izenwasser S (2004) The role of the dopamine transporter in cocaine abuse. Neurotox Res 6:379–383. doi: 10.1007/BF03033312

Jaber M, Robinson SW, Missale C, Caron MG (1996) Dopamine receptors and brain function. Neuropharmacology 35:1503–19.

Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118–34. doi: 10.1210/edrv-12-2-118

Jane Reece, Urry LA, Meyers N, et al (2011) Campbell Biology: 9th edition, Australian version.

Jankovic J, Chen S, Le WD (2005) The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. Prog Neurobiol 77:128–38. doi: 10.1016/j.pneurobio.2005.09.001

Jiang X, Nardelli J (2015) Cellular and molecular introduction to brain development. Neurobiol Dis. doi: 10.1016/j.nbd.2015.07.007

Johnson MH (2001) Functional brain development in humans. Nat Rev Neurosci 2:475–483. doi: 10.1038/35081509

Johnson SA, Fournier NM, Kalynchuk LE (2006) Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. Behav Brain Res 168:280–8. doi: 10.1016/j.bbr.2005.11.019

Juruena MF (2014) Early-life stress and HPA axis trigger recurrent adulthood depression. Epilepsy Behav 38:148–59. doi: 10.1016/j.yebeh.2013.10.020

Kaffman A, Meaney MJ (2007) Neurodevelopmental sequelae of postnatal maternal care in rodents: Clinical and research implications of molecular insights. J Child Psychol Psychiatry Allied Discip 48:224–244. doi: 10.1111/j.1469-7610.2007.01730.x

Kalivas PW, Duffy P (1995) Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. Brain Res 675:325–8.

Kalivas PW, McFarland K (2003) Brain circuitry and the reinstatement of cocaine-seeking behavior. Psychopharmacology (Berl) 168:44–56. doi: 10.1007/s00213-003-1393-2

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. Am J Psychiatry 162:1403–13. doi: 10.1176/appi.ajp.162.8.1403

Kandel ER, Squire LR (2000) Neuroscience: breaking down scientific barriers to the study of brain and mind. Science 290:1113–20.

Kanter JW, Busch AM, Weeks CE, Landes SJ (2008) The nature of clinical depression: symptoms, syndromes, and behavior analysis. Behav Anal 31:1–21.

Katon WJ (2003) Clinical and health services relationships between major depression, depressive symptoms, and general medical illness. Biol Psychiatry 54:216–226. doi: 10.1016/S0006-3223(03)00273-7

Katunar MR, Saez T, Brusco A, Antonelli MC (2009) Immunocytochemical expression of dopamine-related transcription factors Pitx3 and Nurr1 in prenatally stressed adult rats. J Neurosci Res 87:1014–22. doi: 10.1002/jnr.21911

Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8:844–58. doi: 10.1038/nrn2234

Kaufman J, Plotsky PM, Nemeroff CB, Charney DS (2000) Effects of early adverse experiences on brain structure and function: clinical

implications. Biol Psychiatry 48:778–790. doi: 10.1016/S0006-3223(00)00998-7

Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765–76. doi: 10.1016/j.neubiorev.2003.11.015

Kendler KS, Gallagher TJ, Abelson JM, Kessler RC (1996) Lifetime prevalence, demographic risk factors, and diagnostic validity of nonaffective psychosis as assessed in a US community sample. The National Comorbidity Survey. Arch Gen Psychiatry 53:1022–31.

Kendler KS, Gardner CO (2014) Sex differences in the pathways to major depression: a study of opposite-sex twin pairs. Am J Psychiatry 171:426–35. doi: 10.1176/appi.ajp.2013.13101375

Kendler KS, Gatz M, Gardner CO, Pedersen NL (2005) Age at onset and familial risk for major depression in a Swedish national twin sample. Psychol Med 35:1573–9. doi: 10.1017/S0033291705005714

Kern N, Sheldrick AJ, Schmidt FM, Minkwitz J (2012) Neurobiology of depression and novel antidepressant drug targets. Curr Pharm Des 18:5791–801.

Kessler RC (1997) The effects of stressful life events on depression. Annu Rev Psychol 48:191–214. doi: 10.1146/annurev.psych.48.1.191

Kessler RC (2003) Epidemiology of women and depression. J Affect Disord 74:5–13.

Kessler RC, McGonagle KA, Swartz M, et al (1993) Sex and depression in the National Comorbidity Survey. I: Lifetime prevalence, chronicity and recurrence. J Affect Disord 29:85–96.

Khantzian EJ (1985) The self-medication hypothesis of addictive disorders: focus on heroin and cocaine dependence. Am J Psychiatry 142:1259–1264. doi: 10.1176/ajp.142.11.1259

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. doi: 10.1007/s00213-004-1989-1

Kikusui T, Ichikawa S, Mori Y (2009) Maternal deprivation by early weaning increases corticosterone and decreases hippocampal BDNF and neurogenesis in mice. Psychoneuroendocrinology 34:762–72. doi: 10.1016/j.psyneuen.2008.12.009

Kikusui T, Mori Y (2009) Behavioural and neurochemical consequences of early weaning in rodents. J Neuroendocrinol 21:427–31. doi: 10.1111/j.1365-2826.2009.01837.x

Kim J, Inoue K, Ishii J, et al (2007) A MicroRNA feedback circuit in midbrain dopamine neurons. Science 317:1220–4. doi: 10.1126/science.1140481

Kim J, Park B-H, Lee JH, et al (2011) Cell type-specific alterations in the nucleus accumbens by repeated exposures to cocaine. Biol Psychiatry 69:1026–34. doi: 10.1016/j.biopsych.2011.01.013

Kim MJ, Whalen PJ (2009) The Structural Integrity of an Amygdala-Prefrontal Pathway Predicts Trait Anxiety. J Neurosci 29:11614–11618. doi: 10.1523/JNEUROSCI.2335-09.2009

Kishi T, Yoshimura R, Fukuo Y, et al (2012) The serotonin 1A receptor gene confer susceptibility to mood disorders: results from an extended meta-analysis of patients with major depression and bipolar disorder. Eur Arch Psychiatry Clin Neurosci 263:105–118. doi: 10.1007/s00406-012-0337-4

Klein C, Patte-Mensah C, Taleb O, et al (2013) The neuroprotector kynurenic acid increases neuronal cell survival through neprilysin induction. Neuropharmacology 70:254–260. doi: 10.1016/j.neuropharm.2013.02.006

Klein DN, Lewinsohn PM, Rohde P, et al (2002) Clinical features of major depressive disorder in adolescents and their relatives: impact on familial aggregation, implications for phenotype definition, and specificity of transmission. J Abnorm Psychol 111:98–106.

Knowles RG, Moncada S (1994) Nitric oxide synthases in mammals. Biochem J 298 (Pt 2:249–58.

Köhler S, Cierpinsky K, Kronenberg G, Adli M (2015) The serotonergic system in the neurobiology of depression: Relevance for novel antidepressants. J Psychopharmacol. doi: 10.1177/0269881115609072

Kohli MA, Lucae S, Saemann PG, et al (2011) The neuronal transporter gene SLC6A15 confers risk to major depression. Neuron 70:252–65. doi: 10.1016/j.neuron.2011.04.005

Kolb B, Gibb R (2011) Brain plasticity and behaviour in the developing brain. J Can Acad Child Adolesc Psychiatry 20:265–76.

Kolb B, Gibb R (2010) Tactile stimulation after frontal or parietal cortical injury in infant rats facilitates functional recovery and produces synaptic changes in adjacent cortex. Behav Brain Res 214:115–20. doi: 10.1016/j.bbr.2010.04.024

Kolb B, Mychasiuk R, Gibb R (2014) Brain development, experience, and behavior. Pediatr Blood Cancer 61:1720–3. doi: 10.1002/pbc.24908

Koob GF (2008) A role for brain stress systems in addiction. Neuron 59:11–34. doi: 10.1016/j.neuron.2008.06.012

Koob GF (2009a) The role of CRF and CRF-related peptides in the dark side of addiction. Brain Res 1314:3–14. doi: 10.1016/j.brainres.2009.11.008

Koob GF (2009b) Neurobiological substrates for the dark side of compulsivity in addiction. Neuropharmacology 56 Suppl 1:18–31. doi: 10.1016/j.neuropharm.2008.07.043

Koob GF, Le Moal M (2001) Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 24:97–129. doi: 10.1016/S0893-133X(00)00195-0

Koob GF, Le Moal M (2008a) Addiction and the brain antireward system. Annu Rev Psychol 59:29–53. doi: 10.1146/annurev.psych.59.103006.093548

Koob GF, Le Moal M (2008b) Review. Neurobiological mechanisms for opponent motivational processes in addiction. Philos Trans R Soc Lond B Biol Sci 363:3113–23. doi: 10.1098/rstb.2008.0094

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217–238. doi: 10.1038/npp.2010.4

Korosi ABT (2009) The pathways from mother's love to baby's future. Front Behav Neurosci 3:27. doi: 10.3389/neuro.08.027.2009

Kosten TA, Zhang XY, Kehoe P (2003) Chronic neonatal isolation stress enhances cocaine-induced increases in ventral striatal dopamine levels in rat pups. Brain Res Dev Brain Res 141:109–16.

Kravitz A V, Freeze BS, Parker PRL, et al (2010) Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. Nature 466:622–6. doi: 10.1038/nature09159

Krishnan V, Nestler EJ (2008) The molecular neurobiology of depression. Nature 455:894–902. doi: 10.1038/nature07455

Krishnan V, Nestler EJ (2010) Linking molecules to mood: new insight into the biology of depression. Am J Psychiatry 167:1305–20. doi: 10.1176/appi.ajp.2009.10030434

Kronenberg G, Gertz K, Heinz A, Endres M (2014) Of mice and men: modelling post-stroke depression experimentally. Br J Pharmacol 171:4673–89. doi: 10.1111/bph.12775

Kudielka BM, Kirschbaum C (2005) Sex differences in HPA axis responses to stress: a review. Biol Psychol 69:113–32. doi: 10.1016/j.biopsycho.2004.11.009

Kundakovic M, Champagne F a (2014) Early-Life Experience, Epigenetics, and the Developing Brain. Neuropsychopharmacology 40:141–153. doi: 10.1038/npp.2014.140

Lajud N, Roque A, Cajero M, et al (2012) Periodic maternal separation decreases hippocampal neurogenesis without affecting basal corticosterone during the stress hyporesponsive period, but alters HPA axis and coping behavior in adulthood. Psychoneuroendocrinology 37:410–20. doi: 10.1016/j.psyneuen.2011.07.011

Lalanne L, Ayranci G, Kieffer BL, Lutz P-E (2014) The kappa opioid receptor: from addiction to depression, and back. Front psychiatry 5:170. doi: 10.3389/fpsyt.2014.00170

Lambert KG, Gerecke KM, Quadros PS, et al (2000) Activity-stress increases density of GFAP-immunoreactive astrocytes in the rat hippocampus. Stress 3:275–84.

Lanfumey L, Mongeau R, Cohen-Salmon C, Hamon M (2008) Corticosteroid–serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neurosci Biobehav Rev 32:1174–1184. doi: 10.1016/j.neubiorev.2008.04.006

Lashley KS. Brain mechanisms and intelligence: a quantitative study of injuries to the brain. New York: Dover Publications; 1963. 186

Laugeray A, Launay JM, Callebert J, et al (2010) Peripheral and cerebral metabolic abnormalities of the tryptophan-kynurenine pathway in a murine model of major depression. Behav Brain Res 210:84–91. doi: 10.1016/j.bbr.2010.02.014

Lavaur J, Mineur YS, Picciotto MR (2009) The membrane cytoskeletal protein adducin is phosphorylated by protein kinase C in D1 neurons

of the nucleus accumbens and dorsal striatum following cocaine administration. J Neurochem 111:1129–37. doi: 10.1111/j.1471-4159.2009.06405.x

Lavretsky H, Lesser IM, Wohl M, et al (1999) Clinical and neuroradiologic features associated with chronicity in late-life depression. Am J Geriatr Psychiatry 7:309–16.

Le François B, Soo J, Millar AM, et al (2015) Chronic mild stress and antidepressant treatment alter 5-HT1A receptor expression by modifying DNA methylation of a conserved Sp4 site. Neurobiol Dis 82:332–41. doi: 10.1016/j.nbd.2015.07.002

LeDoux JE (1993) Emotional memory systems in the brain. Behav Brain Res 58:69–79. doi: 10.1016/0166-4328(93)90091-4

Lein ES, Hawrylycz MJ, Ao N, et al (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–76. doi: 10.1038/nature05453

Leo D, di Porzio U, Racagni G, et al (2007) Chronic cocaine administration modulates the expression of transcription factors involved in midbrain dopaminergic neuron function. Exp Neurol 203:472–80. doi: 10.1016/j.expneurol.2006.08.024

Leonard B, Maes M (2012) Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. Neurosci Biobehav Rev 36:764–85. doi: 10.1016/j.neubiorev.2011.12.005

Leventopoulos M, Russig H, Feldon J, et al (2009) Neuropharmacology Early deprivation leads to long-term reductions in motivation for reward and 5-HT1A binding and both effects are reversed by fluoxetine. Neuropharmacology 56:692–701. doi: 10.1016/j.neuropharm.2008.12.005

Levine S (1957) Infantile experience and resistance to physiological stress. Science 126:405.

Lewis SJ, Lawlor DA, Davey Smith G, et al (2006) The thermolabile variant of MTHFR is associated with depression in the British Women's Heart and Health Study and a meta-analysis. Mol Psychiatry 11:352–60. doi: 10.1038/sj.mp.4001790

Li Y, Robinson TE, Bhatnagar S (2003) Effects of maternal separation on

behavioural sensitization produced by repeated cocaine administration in adulthood. Brain Res 960:42–7.

Lin JC, Rosenthal A (2003) Molecular mechanisms controlling the development of dopaminergic neurons. Semin Cell Dev Biol 14:175–80.

Liu D, Diorio J, Day JC, et al (2000) Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosci 3:799–806. doi: 10.1038/77702

Liu D, Diorio J, Tannenbaum B, et al (1997) Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277:1659–62.

Liu SQ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405:454–8. doi: 10.1038/35013064

Llorente-Berzal A, Fuentes S, Gagliano H, et al (2011) Sex-dependent effects of maternal deprivation and adolescent cannabinoid treatment on adult rat behaviour. Addict Biol 16:624–37. doi: 10.1111/j.1369-1600.2011.00318.x

Lohoff FW (2010) Overview of the genetics of major depressive disorder. Curr Psychiatry Rep 12:539–46. doi: 10.1007/s11920-010-0150-6

López-Figueroa AL, Norton CS, López-Figueroa MO, et al (2004) Serotonin 5-HT1A, 5-HT1B, and 5-HT2A receptor mRNA expression in subjects with major depression, bipolar disorder, and schizophrenia. Biol Psychiatry 55:225–33.

López-León S, Janssens ACJW, González-Zuloeta Ladd AM, et al (2008) Meta-analyses of genetic studies on major depressive disorder. Mol Psychiatry 13:772–85. doi: 10.1038/sj.mp.4002088

López-Muñoz F, Alamo C (2009) Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. Curr Pharm Des 15:1563–86.

Lopizzo N, Bocchio Chiavetto L, Cattane N, et al (2015) Gene-environment interaction in major depression: focus on experience-dependent biological systems. Front psychiatry 6:68. doi: 10.3389/fpsyt.2015.00068

Lu B, Pang PT, Woo NH (2005a) The yin and yang of neurotrophin action. Nat Rev Neurosci 6:603–614. doi: 10.1038/nrn1726

Lu K, McDaniel AH, Tordoff MG, et al (2005b) No relationship between sequence variation in protein coding regions of the Tas1r3 gene and saccharin preference in rats. Chem Senses 30:231–240. doi: 10.1093/chemse/bji019

Lund TD, Rovis T, Chung WCJ, Handa RJ (2005) Novel actions of estrogen receptor-beta on anxiety-related behaviors. Endocrinology 146:797–807. doi: 10.1210/en.2004-1158

Luo Z, Volkow ND, Heintz N, et al (2011) Acute cocaine induces fast activation of D1 receptor and progressive deactivation of D2 receptor striatal neurons: in vivo optical microprobe [Ca2+]i imaging. J Neurosci 31:13180–90. doi: 10.1523/JNEUROSCI.2369-11.2011

Lupien SJ (2014) The double-hit effect of childhood maltreatment on drug relapse. JAMA psychiatry 71:871–2. doi: 10.1001/jamapsychiatry.2014.924

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445. doi: 10.1038/nrn2639

Lüscher C, Malenka RC (2011) Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. Neuron 69:650–63. doi: 10.1016/j.neuron.2011.01.017

Lykhmus O, Voytenko L, Koval L, et al (2015) α7 Nicotinic acetylcholine receptor-specific antibody induces inflammation and amyloid β42 accumulation in the mouse brain to impair memory. PLoS One 10:e0122706. doi: 10.1371/journal.pone.0122706

Macrì S, Chiarotti F, Würbel H (2008) Maternal separation and maternal care act independently on the development of HPA responses in male rats. Behav Brain Res 191:227–34. doi: 10.1016/j.bbr.2008.03.031

Macrì S, Zoratto F, Laviola G (2011) Early-stress regulates resilience, vulnerability and experimental validity in laboratory rodents through mother-offspring hormonal transfer. Neurosci Biobehav Rev 35:1534–1543. doi: 10.1016/j.neubiorev.2010.12.014

Maes M, Bosmans E, Meltzer HY, et al (1993) Interleukin-1 beta: a putative mediator of HPA axis hyperactivity in major depression? Am J Psychiatry 150:1189–93.

Maes M, Mihaylova I, Kubera M, et al (2009) Increased 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, in major depression and myalgic encephalomyelitis / chronic fatigue syndrome. Neuro Endocrinol Lett 30:715–22.

Malchow B, Strocka S, Frank F, et al (2014) Stereological investigation of the posterior hippocampus in affective disorders. J Neural Transm. doi: 10.1007/s00702-014-1316-x

Maldonado R, Rodríguez de Fonseca F (2002) Cannabinoid addiction: behavioral models and neural correlates. J Neurosci 22:3326–31. doi: 20026358

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285:1870–4.

Marinelli M, Piazza PV (2002) Interaction between glucocorticoid hormones, stress and psychostimulant drugs. Eur J Neurosci 16:387–94.

Marshall PJ, Reeb BC, Fox NA, et al (2008) Effects of early intervention on EEG power and coherence in previously institutionalized children in Romania. Dev Psychopathol 20:861–80. doi: 10.1017/S0954579408000412

Martin M, Ledent C, Parmentier M, et al (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology (Berl) 159:379–387. doi: 10.1007/s00213-001-0946-5

Martini M, Pinto AX, Valverde O (2014) Estrous cycle and sex affect cocaine-induced behavioural changes in CD1 mice. Psychopharmacology (Berl) 231:2647–59. doi: 10.1007/s00213-014-3433-5

Martini M, Valverde O (2011) A single episode of maternal deprivation impairs the motivation for cocaine in adolescent mice. Psychopharmacology (Berl) 219:149–158. doi: 10.1007/s00213-011-2385-2

Mathers CD, Fat DM, Inoue M, et al (2005) Counting the dead and what they died from: an assessment of the global status of cause of death data. Bull World Health Organ 83:171–7. doi: /S0042-96862005000300009

Mathews DC, Henter ID, Zarate CA (2012) Targeting the glutamatergic system to treat major depressive disorder: rationale and progress to

date. Drugs 72:1313–33. doi: 10.2165/11633130-000000000-00000

Matsuzawa J, Matsui M, Konishi T, et al (2001) Age-related volumetric changes of brain gray and white matter in healthy infants and children. Cereb Cortex 11:335–42.

Matsuzawa S, Suzuki T, Misawa M, Nagase H (1999) Involvement of dopamine D(1) and D(2) receptors in the ethanol-associated place preference in rats exposed to conditioned fear stress. Brain Res 835:298–305.

Matthes HWD, Maldonado R, Simonin F, et al (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. Nature 383:819–823. doi: 10.1038/383819a0

Matthews K, Robbins TW (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.

Matthews K, Robbins TW, Everitt BJ, Caine SB (1999) Repeated neonatal maternal separation alters intravenous cocaine self-administration in adult rats. Psychopharmacology (Berl) 141:123–34.

McEwen BS (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87:873–904. doi: 10.1152/physrev.00041.2006

McGowan PO, Suderman M, Sasaki A, et al (2011) Broad epigenetic signature of maternal care in the brain of adult rats. PLoS One 6:e14739. doi: 10.1371/journal.pone.0014739

McGuffin P, Katz R, Watkins S, Rutherford J (1996) A hospital-based twin register of the heritability of DSM-IV unipolar depression. Arch Gen Psychiatry 53:129–36.

McIntosh J, Anisman H, Merali Z (1999) Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. Dev Brain Res 113:97–106. doi: 10.1016/S0165-3806(99)00005-X

McKittrick CR, Magariños AM, Blanchard DC, et al (2000) Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. Synapse 36:85–94. doi: 10.1002/(SICI)1098-2396(200005)36:2<85::AID-

SYN1>3.0.CO;2-Y

McLeod TM, López-Figueroa AL, López-Figueroa MO (2001) Nitric oxide, stress, and depression. Psychopharmacol Bull 35:24–41.

McNally L, Bhagwagar Z, Hannestad J (2008) Inflammation, glutamate, and glia in depression: a literature review. CNS Spectr 13:501–10.

Meaney M, Aitken D, van Berkel C, et al (1988) Effect of neonatal handling on age-related impairments associated with the hippocampus. Science (80-) 239:766–768. doi: 10.1126/science.3340858

Meiser J, Weindl D, Hiller K (2013) Complexity of dopamine metabolism. Cell Commun Signal 11:34. doi: 10.1186/1478-811X-11-34

Melis M, Spiga S, Diana M (2005) The dopamine hypothesis of drug addiction: hypodopaminergic state. Int Rev Neurobiol 63:101–54. doi: 10.1016/S0074-7742(05)63005-X

Mena G, Giraudon I, Álvarez E, et al (2013) Cocaine-related health emergencies in Europe: a review of sources of information, trends and implications for service development. Eur Addict Res 19:74–81. doi: 10.1159/000341719

Merikangas KR, Avenevoli S, Dierker L, Grillon C (1999) Vulnerability factors among children at risk for anxiety disorders. Biol Psychiatry 46:1523–35.

Meyer JS (1983) Early adrenalectomy stimulates subsequent growth and development of the rat brain. Exp Neurol 82:432–46.

Miguel-Hidalgo JJ, Baucom C, Dilley G, et al (2000) Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. Biol Psychiatry 48:861–73.

Miller AH, Maletic V, Raison CL (2009) Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. Biol Psychiatry 65:732–741. doi: 10.1016/j.biopsych.2008.11.029

Miller G, Chen E (2007) Unfavorable socioeconomic conditions in early life presage expression of proinflammatory phenotype in adolescence. Psychosom Med 69:402–9. doi: 10.1097/PSY.0b013e318068fcf9

Miller GE, Cole SW (2012) Clustering of depression and inflammation in adolescents previously exposed to childhood adversity. Biol Psychiatry 72:34–40. doi: 10.1016/j.biopsych.2012.02.034

Millstein R a., Holmes A (2007) Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neurosci Biobehav Rev 31:3–17. doi: 10.1016/j.neubiorev.2006.05.003

Ming G, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223–50. doi: 10.1146/annurev.neuro.28.051804.101459

Missale C, Nash SR, Robinson SW, et al (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189–225.

Molendijk ML, Spinhoven P, Polak M, et al (2014) Serum BDNF concentrations as peripheral manifestations of depression: evidence from a systematic review and meta-analyses on 179 associations (N=9484). Mol Psychiatry 19:791–800. doi: 10.1038/mp.2013.105

Mora S, Dussaubat N, Díaz-Véliz G (1996) Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology 21:609–20.

Moret C, Briley M (2011) The importance of norepinephrine in depression. Neuropsychiatr Dis Treat 7:9–13. doi: 10.2147/NDT.S19619

Morgan D, Grant KA, Gage HD, et al (2002) Social dominance in monkeys: dopamine D2 receptors and cocaine self-administration. Nat Neurosci 5:169–74. doi: 10.1038/nn798

Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681–3.

Mortimer, W.G. (1901) Peru. The History of Coca. "The Divine Plant" of the Incas . New York: J.H. Vail.

Motivala SJ, Sarfatti A, Olmos L, Irwin MR (2005) Inflammatory markers and sleep disturbance in major depression. Psychosom Med 67:187–94. doi: 10.1097/01.psy.0000149259.72488.09

Mounteney J, Griffiths P, Sedefov R, et al (2015) The drug situation in Europe: an overview of data available on illicit drugs and new psychoactive substances from European monitoring in 2015. Addiction. doi: 10.1111/add.13056

Müller N, Schwarz MJ (2007) The immune-mediated alteration of serotonin and glutamate: towards an integrated view of depression. Mol Psychiatry 12:988–1000. doi: 10.1038/sj.mp.4002006

Murray CJL, Lopez AD (2013) Measuring the global burden of disease. N Engl J Med 369:448–57. doi: 10.1056/NEJMra1201534

Murray L, Cooper P (1997a) Effects of postnatal depression on infant development. Arch Dis Child 77:99–101.

Murray L, Cooper PJ (1997b) Postpartum depression and child development. Psychol Med 27:253–260. doi: 10.1017/S0033291796004564

Myers MM, Brunelli SA, Squire JM, et al (1989) Maternal behavior of SHR rats and its relationship to offspring blood pressures. Dev Psychobiol 22:29–53. doi: 10.1002/dev.420220104

Myint A, Ku Y, Verkerk R, et al (2007) Kynurenine pathway in major depression : Evidence of impaired neuroprotection. 98:143–151. doi: 10.1016/j.jad.2006.07.013

Myint AM (2012) Kynurenines: from the perspective of major psychiatric disorders. FEBS J 279:1375–85. doi: 10.1111/j.1742-4658.2012.08551.x

Myint AM, Kim YK (2003) Cytokine-serotonin interaction through IDO: a neurodegeneration hypothesis of depression. Med Hypotheses 61:519–25.

Nader MA, Morgan D, Gage HD, et al (2006) PET imaging of dopamine D2 receptors during chronic cocaine self-administration in monkeys. Nat Neurosci 9:1050–6. doi: 10.1038/nn1737

Navarrete F, Rodríguez-Arias M, Martín-García E, et al (2013) Role of CB2 cannabinoid receptors in the rewarding, reinforcing, and physical effects of nicotine. Neuropsychopharmacology 38:2515–24. doi: 10.1038/npp.2013.157

Nazarian A, Russo SJ, Festa ED, et al (2004) The role of D1 and D2 receptors in the cocaine conditioned place preference of male and female rats. Brain Res Bull 63:295–9. doi: 10.1016/j.brainresbull.2004.03.004

Nemeroff CB, Owens MJ (2002) Treatment of mood disorders. Nat Neurosci 5:1068–1070. doi: 10.1038/nn943

Nestler EJ (2005a) Is there a common molecular pathway for addiction? Nat Neurosci 8:1445–9. doi: 10.1038/nn1578

Nestler EJ (2005b) The neurobiology of cocaine addiction. Sci Pract Perspect 3:4–10.

Nestler EJ (2014) Epigenetic Mechanisms of Depression. JAMA Psychiatry 71:454. doi: 10.1001/jamapsychiatry.2013.4291

Nestler EJ, Barrot M, DiLeone RJ, et al (2002) Neurobiology of depression. Neuron 34:13–25.

Nestler EJ, Carlezon WA (2006) The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 59:1151–9. doi: 10.1016/j.biopsych.2005.09.018

Nestler EJ, Peña CJ, Kundakovic M, et al (2015) Epigenetic Basis of Mental Illness. Neuroscientist. doi: 10.1177/1073858415608147

Nesvåg R, Knudsen GP, Bakken IJ, et al (2015) Substance use disorders in schizophrenia, bipolar disorder, and depressive illness: a registry-based study. Soc Psychiatry Psychiatr Epidemiol 50:1267–76. doi: 10.1007/s00127-015-1025-2

Nishi M, Horii-Hayashi N, Sasagawa T, Matsunaga W (2013) Effects of early life stress on brain activity: implications from maternal separation model in rodents. Gen Comp Endocrinol 181:306–9. doi: 10.1016/j.ygcen.2012.09.024

Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7:697–709. doi: 10.1038/nrn1970

Nolen-Hoeksema S (2001) Gender Differences in Depression. Curr Dir Psychol Sci 10:173–176. doi: 10.1111/1467-8721.00142

Nolen-Hoeksema S, Larson J, Grayson C (1999) Explaining the gender difference in depressive symptoms. J Pers Soc Psychol 77:1061–72.

Nutt DJ (2008) Relationship of neurotransmitters to the symptoms of major depressive disorder. J Clin Psychiatry 69 Suppl E:4–7.

O' Mahony SM, Clarke G, Dinan TG, Cryan JF (2015) Early Life Adversity and Brain Development: Is the Microbiome a Missing Piece of the Puzzle? Neuroscience. doi: 10.1016/j.neuroscience.2015.09.068

O'Connor RM, Moloney RD, Glennon J, et al (2015) Enhancing glutamatergic transmission during adolescence reverses early-life stress-induced deficits in the rewarding effects of cocaine in rats. Neuropharmacology 99:168–176. doi: 10.1016/j.neuropharm.2015.07.012

Ojeda V, Fuentealba JA, Galleguillos D, Andrés ME (2003) Rapid increase of Nurr1 expression in the substantia nigra after 6-hydroxydopamine lesion in the striatum of the rat. J Neurosci Res 73:686–97. doi: 10.1002/jnr.10705

Okuda S, Nishiyama N, Saito H, Katsuki H (1998) 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. J Neurochem 70:299–307.

Osterlund MK, Witt M-R, Gustafsson J-A (2005) Estrogen action in mood and neurodegenerative disorders: estrogenic compounds with selective properties-the next generation of therapeutics. Endocrine 28:235–42. doi: 10.1385/ENDO:28:3:235

Oswald LM, Wong DF, McCaul M, et al (2005) Relationships Among Ventral Striatal Dopamine Release, Cortisol Secretion, and Subjective Responses to Amphetamine. Neuropsychopharmacology 30:821–32. doi: 10.1038/sj.npp.1300667

Own LS, Patel PD (2013) Maternal behavior and offspring resiliency to maternal separation in c57bl/6 mice. Horm Behav 63:411–417. doi: 10.1016/j.yhbeh.2012.11.010

Pacak K, Tjurmina O, Palkovits M, et al (2002) Chronic hypercortisolemia inhibits dopamine synthesis and turnover in the nucleus accumbens: an in vivo microdialysis study. Neuroendocrinology 76:148–57. doi: 64522

Pace TWW, Mletzko TC, Alagbe O, et al (2006) Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. Am J Psychiatry 163:1630–3. doi: 10.1176/appi.ajp.163.9.1630

Palacios JM, Waeber C, Hoyer D, Mengod G (1990) Distribution of serotonin receptors. Ann N Y Acad Sci 600:36–52.

Palanza P (2001) Animal models of anxiety and depression: how are females different? Neurosci Biobehav Rev 25:219–33.

Palanza P, Gioiosa L, Parmigiani S (2001) Social stress in mice: gender differences and effects of estrous cycle and social dominance. Physiol Behav 73:411–20.

Palazidou E (2012) The neurobiology of depression. Br Med Bull 101:127–145. doi: 10.1093/bmb/lds004

Papp M, Willner P, Muscat R (1991) An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. Psychopharmacology (Berl) 104:255–9.

Parker KJ, Schatzberg AF, Lyons DM (2003) Neuroendocrine aspects of hypercortisolism in major depression. Horm Behav 43:60–6.

Pascoli V, Turiault M, Lüscher C (2012) Reversal of cocaine-evoked synaptic potentiation resets drug-induced adaptive behaviour. Nature 481:71–5. doi: 10.1038/nature10709

Pasquini M, Berardelli I, Biondi M (2014) Ethiopathogenesis of depressive disorders. Clin Pract Epidemiol Ment Health 10:166–71. doi: 10.2174/1745017901410010166

Paterson NE, Fedolak A, Olivier B, et al (2010) Psychostimulant-like discriminative stimulus and locomotor sensitization properties of the wake-promoting agent modafinil in rodents. Pharmacol Biochem Behav 95:449–456. doi: 10.1016/j.pbb.2010.03.006

Paus T, Collins DL, Evans AC, et al (2001) Maturation of white matter in the human brain: a review of magnetic resonance studies. Brain Res Bull 54:255–66.

Paxinos G, Franklin KBJ (2004) The mouse brain in stereotaxic coordinates.

Pearson TA, Manolio TA (2008) How to interpret a genome-wide association study. JAMA 299:1335–44. doi: 10.1001/jama.299.11.1335

Pechtel P, Pizzagalli D a. (2011) Effects of early life stress on cognitive and affective function: An integrated review of human literature. Psychopharmacology (Berl) 214:55–70. doi: 10.1007/s00213-010-2009-2

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–67.

Phelps EA (2004) Human emotion and memory: interactions of the amygdala and hippocampal complex. Curr Opin Neurobiol 14:198–202. doi: 10.1016/j.conb.2004.03.015

Piccinelli M, Wilkinson G (2000) Gender differences in depression. Critical review. Br J Psychiatry 177:486–92.

Pierce RC, Kumaresan V (2006) The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? Neurosci Biobehav Rev 30:215–238. doi: 10.1016/j.neubiorev.2005.04.016

Pijlman FT., Wolterink G, Van Ree JM (2003) Physical and emotional stress have differential effects on preference for saccharine and open field behaviour in rats. Behav Brain Res 139:131–138. doi: 10.1016/S0166-4328(02)00124-9

Pittenger C, Duman RS (2008) Stress, depression, and neuroplasticity: a convergence of mechanisms. Neuropsychopharmacology 33:88–109. doi: 10.1038/sj.npp.1301574

Pizzagalli DA (2014) Depression, stress, and anhedonia: toward a synthesis and integrated model. Annu Rev Clin Psychol 10:393–423. doi: 10.1146/annurev-clinpsy-050212-185606

Planeta CS, Marin MT (2002) Effect of cocaine on periadolescent rats with or without early maternal separation. Braz J Med Biol Res 35:1367–71.

Plotsky PM, Meaney MJ (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res 18:195–200.

Polter AM, Kauer JA (2014) Stress and VTA synapses: implications for addiction and depression. Eur J Neurosci 39:1179–88. doi: 10.1111/ejn.12490

Powell K (2006) Neurodevelopment: how does the teenage brain work? Nature 442:865–7. doi: 10.1038/442865a

Powell TR, Fernandes C, Schalkwyk LC (2011) Current Protocols in Mouse Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA

Prins J, Olivier B, Korte SM (2011) Triple reuptake inhibitors for treating subtypes of major depressive disorder: the monoamine hypothesis revisited. Expert Opin Investig Drugs 20:1107–30. doi:

10.1517/13543784.2011.594039

Prus AJ, James JR, Rosecrans JA (2009) Conditioned Place Preference.

Pryce CR, Bettschen D, Feldon J (2001) Comparison of the effects of early handling and early deprivation on maternal care in the rat. Dev Psychobiol 38:239–251. doi: 10.1002/dev.1018

Puglisi-Allegra S, Kempf E, Schleef C, Cabib S (1991) Repeated stressful experiences differently affect brain dopamine receptor subtypes. Life Sci 48:1263–8.

Pulvirenti L, Koob GF (1993) Lisuride reduces psychomotor retardation during withdrawal from chronic intravenous amphetamine self-administration in rats. Neuropsychopharmacology 8:213–8. doi: 10.1038/npp.1993.23

Raedler TJ (2011) Inflammatory mechanisms in major depressive disorder. Curr Opin Psychiatry 24:1. doi: 10.1097/YCO.0b013e32834b9db6

Rajkowska G, Miguel-Hidalgo JJ (2007) Gliogenesis and glial pathology in depression. CNS Neurol Disord Drug Targets 6:219–33.

Rappeneau V, Morel A, Yacoubi M El, et al (2015) Enhanced Cocaine-Associated Contextual Learning in Female H / Rouen Mice Selectively Bred for Depressive-Like Behaviors : Molecular and Neuronal Correlates. 1–12. doi: 10.1093/ijnp/pyv022

Raznahan A, Lerch JP, Lee N, et al (2011) Patterns of coordinated anatomical change in human cortical development: a longitudinal neuroimaging study of maturational coupling. Neuron 72:873–84. doi: 10.1016/j.neuron.2011.09.028

Reddy SDN, Rayala SK, Ohshiro K, et al (2011) Multiple coregulatory control of tyrosine hydroxylase gene transcription. Proc Natl Acad Sci U S A 108:4200–5. doi: 10.1073/pnas.1101193108

Regier DA, Farmer ME, Rae DS, et al (1990) Comorbidity of mental disorders with alcohol and other drug abuse. Results from the Epidemiologic Catchment Area (ECA) Study. JAMA 264:2511–8.

Reichenberg A, Yirmiya R, Schuld A, et al (2001) Cytokine-associated emotional and cognitive disturbances in humans. Arch Gen Psychiatry 58:445–452. doi: 10.1001/archpsyc.58.5.445

Renoir T, Zajac MS, Du X, et al (2011) Sexually dimorphic serotonergic

dysfunction in a mouse model of Huntington's disease and depression. PLoS One 6:e22133. doi: 10.1371/journal.pone.0022133

Réus GZ, Fries GR, Stertz L, et al (2015a) The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders. Neuroscience 300:141–54. doi: 10.1016/j.neuroscience.2015.05.018

Réus GZ, Nacif MP, Abelaira HM, et al (2015b) Ketamine ameliorates depressive-like behaviors and immune alterations in adult rats following maternal deprivation. Neurosci Lett 584:83–7. doi: 10.1016/j.neulet.2014.10.022

Rezayof A, Zarrindast M-R, Sahraei H, Haeri-Rohani A-H-R (2002) Involvement of dopamine D2 receptors of the central amygdala on the acquisition and expression of morphine-induced place preference in rat. Pharmacol Biochem Behav 74:187–97.

Rhodes ME, Rubin RT (1999) Functional sex differences (’sexual diergism') of central nervous system cholinergic systems, vasopressin, and hypothalamic-pituitary-adrenal axis activity in mammals: a selective review. Brain Res Brain Res Rev 30:135–52.

Rice CJ, Sandman CA, Lenjavi MR, Baram TZ (2008) A novel mouse model for acute and long-lasting consequences of early life stress. Endocrinology 149:4892–900. doi: 10.1210/en.2008-0633

Riggs PD (1998) Clinical approach to treatment of ADHD in adolescents with substance use disorders and conduct disorder. J Am Acad Child Adolesc Psychiatry 37:331–2.

Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237:1219–23.

Robbins TW, Everitt BJ (1996) Neurobehavioural mechanisms of reward and motivation. Curr Opin Neurobiol 6:228–236. doi: 10.1016/S0959-4388(96)80077-8

Roberts AJ, McDonald JS, Heyser CJ, et al (2000) {micro}-Opioid Receptor Knockout Mice Do Not Self-Administer Alcohol. J Pharmacol Exp Ther 293:1002–1008.

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev 18:247–91.

Robinson TE, Berridge KC (2001) Incentive-sensitization and addiction.

Addiction 96:103–14. doi: 10.1080/09652140020016996

Rodríguez-Arias M, Montagud-Romero S, Rubio-Araiz A, et al (2015) 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. doi: 10.1111/adb.12301

Roiser JP, Levy J, Fromm SJ, et al (2008) The effect of acute tryptophan depletion on the neural correlates of emotional processing in healthy volunteers. Neuropsychopharmacology 33:1992–2006. doi: 10.1038/sj.npp.1301581

Roque A, Ochoa-Zarzosa A, Torner L (2015) Maternal separation activates microglial cells and induces an inflammatory response in the hippocampus of male rat pups, independently of hypothalamic and peripheral cytokine levels. Brain Behav Immun. doi: 10.1016/j.bbi.2015.09.017

Ros-Simó C, Ruiz-Medina J, Valverde O (2012) Behavioural and neuroinflammatory effects of the combination of binge ethanol and MDMA in mice. Psychopharmacology (Berl) 221:511–25. doi: 10.1007/s00213-011-2598-4

Ros-Simó C, Valverde O (2012) Early-life social experiences in mice affect emotional behaviour and hypothalamic-pituitary-adrenal axis function. Pharmacol Biochem Behav 102:434–41. doi: 10.1016/j.pbb.2012.06.001

Roux S, Froger C, Porsolt RD, et al (2001) Current Protocols in Neuroscience. John Wiley & Sons, Inc., Hoboken, NJ, USA

Roy A, Linnoila M, Karoum F, Pickar D (1986) Relative activity of metabolic pathways for norepinephrine in endogenous depression. Acta Psychiatr Scand 73:624–8.

Ruhé HG, Mason NS, Schene AH (2007) Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. Mol Psychiatry 12:331–59. doi: 10.1038/sj.mp.4001949

Rush AJ, Giles DE, Schlesser MA, et al (1996) The dexamethasone suppression test in patients with mood disorders. J Clin Psychiatry 57:470–84.

Russo SJ, Nestler EJ (2013) The brain reward circuitry in mood disorders. Nat Rev Neurosci 14:609–25. doi: 10.1038/nrn3381

Saavedra A, Giralt A, Arumí H, et al (2013) Regulation of Hippocampal cGMP Levels as a Candidate to Treat Cognitive Deficits in Huntington's Disease. PLoS One 8:1–10. doi: 10.1371/journal.pone.0073664

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10:1110–5. doi: 10.1038/nn1969

Samuels ER, Szabadi E (2008) Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation. Curr Neuropharmacol 6:235–53. doi: 10.2174/157015908785777229

Sas K, Robotka H, Toldi J, Vécsei L (2007) Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. J Neurol Sci 257:221–239. doi: 10.1016/j.jns.2007.01.033

Saveanu R V, Nemeroff CB (2012) Etiology of depression: genetic and environmental factors. Psychiatr Clin North Am 35:51–71. doi: 10.1016/j.psc.2011.12.001

Sayler A, Salmon M (1969) Communal nursing in mice: influence of multiple mothers on the growth of the young. Science 164:1309–10.

Schaechter JD, Wurtman RJ (1990) Serotonin release varies with brain tryptophan levels. Brain Res 532:203–10.

Schafer DP, Lehrman EK, Kautzman AG, et al (2012) Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74:691–705. doi: 10.1016/j.neuron.2012.03.026

Schneiderman N, Ironson G, Siegel SD (2005) Stress and health: psychological, behavioral, and biological determinants. Annu Rev Clin Psychol 1:607–28. doi: 10.1146/annurev.clinpsy.1.102803.144141

Schweizer MC, Henniger MSH, Sillaber I (2009) Chronic mild stress (CMS) in mice: of anhedonia, "anomalous anxiolysis" and activity. PLoS One 4:e4326. doi: 10.1371/journal.pone.0004326

Shalev U, Kafkafi N (2002) Repeated maternal separation does not alter sucrose-reinforced and open-field behaviors. Pharmacol Biochem Behav 73:115–122. doi: 10.1016/S0091-3057(02)00756-6

Sharma HS, Zimmer C, Westman J, Cervós-Navarro J (1992) Acute systemic heat stress increases glial fibrillary acidic protein

immunoreactivity in brain: experimental observations in conscious normotensive young rats. Neuroscience 48:889–901.

Sharples SA, Koblinger K, Humphreys JM, Whelan PJ (2014) Dopamine: a parallel pathway for the modulation of spinal locomotor networks. Front Neural Circuits 8:55. doi: 10.3389/fncir.2014.00055

Shaw P, Greenstein D, Lerch J, et al (2006) Intellectual ability and cortical development in children and adolescents. Nature 440:676–9. doi: 10.1038/nature04513

Sherin JE, Nemeroff CB (2011) Post-traumatic stress disorder: the neurobiological impact of psychological trauma. Dialogues Clin Neurosci 13:263–78.

Shi M, Hu J, Dong X, et al (2008) Association of unipolar depression with gene polymorphisms in the serotonergic pathways in Han Chinese. Acta Neuropsychiatr 20:139–44. doi: 10.1111/j.1601-5215.2008.00282.x

Shimamoto A, Holly EN, Boyson CO, et al (2015) Individual differences in anhedonic and accumbal dopamine responses to chronic social stress and their link to cocaine self-administration in female rats. Psychopharmacology (Berl) 232:825–34. doi: 10.1007/s00213-014-3725-9

Simeone A (2005) Genetic control of dopaminergic neuron differentiation. Trends Neurosci 28:62–5; discussion 65–6. doi: 10.1016/j.tins.2004.11.007

Simonin F (1998) Disruption of the kappa -opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa -agonist U-50,488H and attenuates morphine withdrawal. EMBO J 17:886–897. doi: 10.1093/emboj/17.4.886

Sinha R (2008) Chronic stress, drug use, and vulnerability to addiction. Ann N Y Acad Sci 1141:105–30. doi: 10.1196/annals.1441.030

Sinha R, Talih M, Malison R, et al (2003) Hypothalamic-pituitary-adrenal axis and sympatho-adreno-medullary responses during stress-induced and drug cue-induced cocaine craving states. Psychopharmacology (Berl) 170:62–72. doi: 10.1007/s00213-003-1525-8

Smidt MP, van Schaick HS, Lanctôt C, et al (1997) A homeodomain gene

Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. Proc Natl Acad Sci U S A 94:13305–10.

Smoller JW (2015) The Genetics of Stress-Related Disorders: PTSD, Depression and Anxiety Disorders. Neuropsychopharmacology. doi: 10.1038/npp.2015.266

Sobin C, Sackeim HA (1997) Psychomotor symptoms of depression. Am J Psychiatry 154:4–17.

Sokoloff P, Guillin O, Diaz J, et al (2002) Brain-derived neurotrophic factor controls dopamine D3 receptor expression: implications for neurodevelopmental psychiatric disorders. Neurotox Res 4:671–678. doi: 10.1080/1029842021000045499

Solomon J, George C (1999) Conceptualizations of disorganization in the preschool years: An integration. In: Attachment disorganization. p 213

Somaini L, Donnini C, Manfredini M, et al (2011) Adverse childhood experiences (ACEs), genetic polymorphisms and neurochemical correlates in experimentation with psychotropic drugs among adolescents. Neurosci Biobehav Rev 35:1771–8. doi: 10.1016/j.neubiorev.2010.11.008

Soria G, Castañé A, Ledent C, et al (2006) The lack of A2A adenosine receptors diminishes the reinforcing efficacy of cocaine. Neuropsychopharmacology 31:978–87. doi: 10.1038/sj.npp.1300876

Soria G, Mendizábal V, Touriño C, et al (2005) Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 30:1670–80. doi: 10.1038/sj.npp.1300707

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417–63.

Spiteri T, Musatov S, Ogawa S, et al (2010) The role of the estrogen receptor alpha in the medial amygdala and ventromedial nucleus of the hypothalamus in social recognition, anxiety and aggression. Behav Brain Res 210:211–20. doi: 10.1016/j.bbr.2010.02.033

Steimer T (2002) The biology of fear- and anxiety-related behaviors. Dialogues Clin Neurosci 4:231–49.

Steiner J, Walter M, Gos T, et al (2011) Severe depression is associated with increased microglial quinolinic acid in subregions of the

anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? J Neuroinflammation 8:94. doi: 10.1186/1742-2094-8-94

Sterling P (2012) Allostasis: a model of predictive regulation. Physiol Behav 106:5–15. doi: 10.1016/j.physbeh.2011.06.004

Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 85:367–70.

Stiles J (2008) The fundamentals of brain development: Integrating nature and nurture.

Stiles J, Jernigan TL (2010) The basics of brain development. Neuropsychol Rev 20:327–348. doi: 10.1007/s11065-010-9148-4

Stolberg VB (2011) The use of coca: prehistory, history, and ethnography. J Ethn Subst Abuse 10:126–46. doi: 10.1080/15332640.2011.573310

Stone TW (2001) Endogenous neurotoxins from tryptophan. Toxicon 39:61–73.

Stoof JC, Kebabian JW (1984) Two dopamine receptors: biochemistry, physiology and pharmacology. Life Sci 35:2281–96.

Strekalova T, Spanagel R, Bartsch D, et al (2004) Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. Neuropsychopharmacology 29:2007–17. doi: 10.1038/sj.npp.1300532

Strekalova T, Spanagel R, Dolgov O, Bartsch D (2005) Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. Behav Pharmacol 16:171–80.

Stuart MJ, Baune BT (2014) Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies. Neurosci Biobehav Rev 42:93–115. doi: 10.1016/j.neubiorev.2014.02.001

Sudai E, Croitoru O, Shaldubina A, et al (2011) High cocaine dosage decreases neurogenesis in the hippocampus and impairs working memory. Addict Biol 16:251–60. doi: 10.1111/j.1369-1600.2010.00241.x

Sukoff Rizzo SJ, Neal SJ, Hughes Z a, et al (2012) Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like

phenotypes. Transl Psychiatry 2:1–12. doi: 10.1038/tp.2012.120

Sullivan TN, Farrell AD, Kliewer W (2006) Peer victimization in early adolescence: association between physical and relational victimization and drug use, aggression, and delinquent behaviors among urban middle school students. Dev Psychopathol 18:119–37. doi: 10.1017/S095457940606007X

Suzuki E, Yagi G, Nakaki T, et al (2001) Elevated plasma nitrate levels in depressive states. J Affect Disord 63:221–4.

Swaab DF, Bao A-M, Lucassen PJ (2005) The stress system in the human brain in depression and neurodegeneration. Ageing Res Rev 4:141–94. doi: 10.1016/j.arr.2005.03.003

Tau GZ, Peterson BS (2010) Normal development of brain circuits. Neuropsychopharmacology 35:147–168. doi: 10.1038/npp.2009.115

Taylor HG, Alden J (1997) Age-related differences in outcomes following childhood brain insults: an introduction and overview. J Int Neuropsychol Soc 3:555–67.

Teicher MH, Andersen SL, Polcari A, et al (2003) The neurobiological consequences of early stress and childhood maltreatment. Neurosci Biobehav Rev 27:33–44.

Teicher M.H, Anderson CM, Ohashi K, et al (2014) Childhood maltreatment: altered network centrality of cingulate, precuneus, temporal pole and insula. Biological Psychiatry. 76(4):297-305.

Thanos PK, Volkow ND, Freimuth P, et al (2001) Overexpression of dopamine D2 receptors reduces alcohol self-administration. J Neurochem 78:1094–103.

Thomas AW, Caporale N, Wu C, Wilbrecht L (2015) Early maternal separation impacts cognitive flexibility at the age of first independence in mice. Dev Cogn Neurosci. doi: 10.1016/j.dcn.2015.09.005

Thomas C, Marcaletti S, Feige JN (2011) Current Protocols in Mouse Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA

Thomsen M, Caine SB (2005) Chronic intravenous drug self-administration in rats and mice. Curr Protoc Neurosci Chapter 9:Unit 9.20. doi: 10.1002/0471142301.ns0920s32

Tomihara K, Soga T, Nomura M, et al (2009) Effect of ER-beta gene

disruption on estrogenic regulation of anxiety in female mice. Physiol Behav 96:300–6. doi: 10.1016/j.physbeh.2008.10.014

Tourino C, Valjent E, Ruiz-Medina J, et al (2012) The orphan receptor GPR3 modulates the early phases of cocaine reinforcement. Br J Pharmacol 167:892–904. doi: 10.1111/j.1476-5381.2012.02043.x

Touriño C, Zimmer A, Valverde O (2010) THC Prevents MDMA Neurotoxicity in Mice. PLoS One 5:e9143. doi: 10.1371/journal.pone.0009143

Trajkovska V, Kirkegaard L, Krey G, et al (2009) Activation of glucocorticoid receptors increases 5-HT2A receptor levels. Exp Neurol 218:83–91. doi: 10.1016/j.expneurol.2009.04.008

Tsankova NM, Berton O, Renthal W, et al (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9:519–25. doi: 10.1038/nn1659

Tseng KY, Roubert C, Do L, et al (2000) Selective increase of Nurr1 mRNA expression in mesencephalic dopaminergic neurons of D2 dopamine receptor-deficient mice. Brain Res Mol Brain Res 80:1–6.

Tsivos Z-L, Calam R, Sanders MR, Wittkowski A (2015) Interventions for postnatal depression assessing the mother-infant relationship and child developmental outcomes: a systematic review. Int J Womens Health 7:429–47. doi: 10.2147/IJWH.S75311

Tsuji M, Takeda H, Matsumiya T (2003) Modulation of passive avoidance in mice by the 5-HT1A receptor agonist flesinoxan: comparison with the benzodiazepine receptor agonist diazepam. Neuropsychopharmacology 28:664–674. doi: 10.1038/sj.npp.1300080

Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 411:583–7. doi: 10.1038/35079077

Valverde O, Fournie-Zaluski MC, Roques BP, Maldonado R (1996) The CCKB antagonist PD-134,308 facilitates rewarding effects of endogenous enkephalins but does not induce place preference in rats. Psychopharmacology (Berl) 123:119–26.

Valverde O, Karsak M, Zimmer A (2005) Analysis of the endocannabinoid system by using CB1 cannabinoid receptor knockout mice. Handb Exp Pharmacol 117–45.

Valverde O, Smadja C, Roques BP, Maldonado R (1997) The attenuation of morphine-conditioned place preference following chronic mild stress is reversed by a CCKB receptor antagonist. Psychopharmacology (Berl) 131:79–85.

Valverde O, Torrens M (2012) CB1 receptor-deficient mice as a model for depression. Neuroscience 204:193–206. doi: 10.1016/j.neuroscience.2011.09.031

Van Dam NT, Rando K, Potenza MN, et al (2014) Childhood maltreatment, altered limbic neurobiology, and substance use relapse severity via trauma-specific reductions in limbic gray matter volume. JAMA psychiatry 71:917–25. doi: 10.1001/jamapsychiatry.2014.680

van Harmelen A-L, van Tol M-J, van der Wee NJA, et al (2010) Reduced medial prefrontal cortex volume in adults reporting childhood emotional maltreatment. Biol Psychiatry 68:832–8. doi: 10.1016/j.biopsych.2010.06.011

van Oers HJJ, Ronald de Kloet E, Levine S (1997) Persistent, but Paradoxical, Effects on HPA Regulation of Infants Maternally Deprived at Different Ages. Stress Int J Biol Stress 1:249–261. doi: 10.3109/10253899709013745

Vazquez V, Weiss S, Giros B, et al (2007) Maternal deprivation and handling modify the effect of the dopamine D3 receptor agonist, BP 897 on morphine-conditioned place preference in rats. Psychopharmacology (Berl) 193:475–86. doi: 10.1007/s00213-007-0789-9

Villanueva R (2013) Neurobiology of major depressive disorder. Neural Plast 2013:873278. doi: 10.1155/2013/873278

Volkow ND (2004) The reality of comorbidity: depression and drug abuse. Biol Psychiatry 56:714–7. doi: 10.1016/j.biopsych.2004.07.007

Volkow ND, Baler RD (2014) Addiction science: Uncovering neurobiological complexity. Neuropharmacology 76 Pt B:235–49. doi: 10.1016/j.neuropharm.2013.05.007

Volkow ND, Fowler JS (2000) Addiction, a disease of compulsion and drive: involvement of the orbitofrontal cortex. Cereb Cortex 10:318–25.

Volkow ND, Li T-K (2004) Science and Society: Drug addiction: the

neurobiology of behaviour gone awry. Nat Rev Neurosci 5:963–970. doi: 10.1038/nrn1539

Volkow ND, Morales M (2015) The Brain on Drugs: From Reward to Addiction. Cell 162:712–725. doi: 10.1016/j.cell.2015.07.046

Volkow ND, Wang GJ, Fowler JS, et al (1999) Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors. J Pharmacol Exp Ther 291:409–15.

Volkow ND, Wang G-J, Fowler JS, et al (2002) Brain DA D2 receptors predict reinforcing effects of stimulants in humans: replication study. Synapse 46:79–82. doi: 10.1002/syn.10137

von Knorring A-L (1983) An Adoption Study of Depressive Disorders and Substance Abuse. Arch Gen Psychiatry 40:943. doi: 10.1001/archpsyc.1983.01790080025003

Vorhees C V, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1:848–58. doi: 10.1038/nprot.2006.116

Wang J, Jing L, Toledo-Salas J-C, Xu L (2015) Rapid-onset antidepressant efficacy of glutamatergic system modulators: the neural plasticity hypothesis of depression. Neurosci Bull 31:75–86. doi: 10.1007/s12264-014-1484-6

Wang L, Jiao J, Dulawa SC (2011) Infant maternal separation impairs adult cognitive performance in BALB/cJ mice. Psychopharmacology (Berl) 216:207–218. doi: 10.1007/s00213-011-2209-4

Watson RE, Desesso JM, Hurtt ME, Cappon GD (2006) Postnatal growth and morphological development of the brain: a species comparison. Birth Defects Res B Dev Reprod Toxicol 77:471–84. doi: 10.1002/bdrb.20090

Weaver SA, Diorio J, Meaney MJ (2007) Maternal separation leads to persistent reductions in pain sensitivity in female rats. J Pain 8:962–9. doi: 10.1016/j.jpain.2007.07.001

Webb SJ, Monk CS, Nelson CA (2001) Mechanisms of postnatal neurobiological development: implications for human development. Dev Neuropsychol 19:147–71. doi: 10.1207/S15326942DN1902_2

Weinshenker D, Holmes P V (2015) Regulation of neurological and neuropsychiatric phenotypes by locus coeruleus-derived galanin.

Brain Res. doi: 10.1016/j.brainres.2015.11.025

Weiss RD, Griffin ML, Mirin SM (2009) Drug Abuse as Self-Medication for Depression: An Empirical Study.

Weissman MM, Bland R, Joyce PR, et al (1996) Sex differences in rates of depression: cross-national perspectives. J Affect Disord 29:77–84.

Weller RA, Weller EB, Fristad MA, Bowes JM (1991) Depression in recently bereaved prepubertal children. Am J Psychiatry 148:1536–40.

WHO Report. Depression. 2015.

WHO Report. Global status report on alcohol and health. 2014.

White DA, Birkle DL (2001) The differential effects of prenatal stress in rats on the acoustic startle reflex under baseline conditions and in response to anxiogenic drugs. Psychopharmacology (Berl) 154:169–76.

Widom CS, DuMont K, Czaja SJ (2007) A prospective investigation of major depressive disorder and comorbidity in abused and neglected children grown up. Arch Gen Psychiatry 64:49–56. doi: 10.1001/archpsyc.64.1.49

Willner P, Scheel-Krüger J, Belzung C (2013) The neurobiology of depression and antidepressant action. Neurosci Biobehav Rev 37:2331–71. doi: 10.1016/j.neubiorev.2012.12.007

Willner P, Towell A, Sampson D, et al (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl) 93:358–64.

Winstock AR, Griffiths P, Stewart D (2001) Drugs and the dance music scene: a survey of current drug use patterns among a sample of dance music enthusiasts in the UK. Drug Alcohol Depend 64:9–17.

Wise RA, Koob GF (2014) The development and maintenance of drug addiction. Neuropsychopharmacology 39:254–62. doi: 10.1038/npp.2013.261

Witt ED (2007) Puberty, hormones, and sex differences in alcohol abuse and dependence. Neurotoxicol Teratol 29:81–95. doi: 10.1016/j.ntt.2006.10.013

Wolf ME (2002) Addiction: making the connection between behavioral changes and neuronal plasticity in specific pathways. Mol Interv 2:146–57. doi: 10.1124/mi.2.3.146

World Drug Report (UNODC) 2015.

Xiao Q, Castillo SO, Nikodem VM (1996) Distribution of messenger RNAs for the orphan nuclear receptors Nurr1 and Nur77 (NGFI-B) in adult rat brain using in situ hybridization. Neuroscience 75:221–30.

Xue X, Shao S, Wang W, Shao F (2013) Maternal separation induces alterations in reversal learning and brain-derived neurotrophic factor expression in adult rats. Neuropsychobiology 68:243–9. doi: 10.1159/000356188

Yoshida T, Ishikawa M, Niitsu T, et al (2012) Decreased serum levels of mature brain-derived neurotrophic factor (BDNF), but not its precursor proBDNF, in patients with major depressive disorder. PLoS One 7:e42676. doi: 10.1371/journal.pone.0042676

You I-J, Wright SR, Garcia-Garcia AL, et al (2015) 5-HT1A Autoreceptors in the Dorsal Raphe Nucleus Convey Vulnerability to Compulsive Cocaine Seeking. Neuropsychopharmacology. doi: 10.1038/npp.2015.268

Zeanah CH, Nelson CA, Fox NA, et al (2003) Designing research to study the effects of institutionalization on brain and behavioral development: the Bucharest Early Intervention Project. Dev Psychopathol 15:885–907.

Zhang T-Y, Hellstrom IC, Bagot RC, et al (2010) Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. J Neurosci 30:13130–7. doi: 10.1523/JNEUROSCI.1039-10.2010

Zhang T-Y, Meaney MJ (2010) Epigenetics and the environmental regulation of the genome and its function. Annu Rev Psychol 61:439–66, C1–3. doi: 10.1146/annurev.psych.60.110707.163625

Zimmerberg B, Farley MJ (1993) Sex differences in anxiety behavior in rats: role of gonadal hormones. Physiol Behav 54:1119–24.

WEBSITES

Mental Health Strategy of the National Health System, 2009-2013 Ministry of Health

http://www.msssi.gob.es/organizacion/sns/planCalidadSNS/docs/saludmental/SaludMental2009-2013.pdf

Ministry of Health, Press release, 2015

http://www.msssi.gob.es/gabinete/notasPrensa.do?id=3581

Harrison Narcotics Tax Act, 1974

http://www.druglibrary.org/schaffer/history/e1910/harrisonact.htm

ANNEX

Article 1

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 Feb 4;65:104-17. doi: 10.1016/j.pnpbp.2015.09.003

Article 2

Maternal separation alters cocaine addiction behaviour through dopaminergic system.

Gracia-Rubio I[1], Martinez-Laorden E[2,3], Moscoso-Castro M[1], Milanés MV[2,3], Laorden ML[2,3], Valverde O[1,4]

[1] Neurobiology of Behavior Research Group (GReNeC). Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

[2]Group of Cellular and Molecular Pharmacology, Faculty of Medicine, University of Murcia, Murcia, Spain.

[3]Instituto Murciano de Investigación Biosanitaria (IMIB), Murcia, Spain

[4]Neuroscience Research Program. IMIM (Hospital del Mar Research Institute) Barcelona, Spain.

*** Corresponding author at:**

Olga Valverde MD PhD

Neurobiology of Behavior Research Group (GReNeC)
Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona.
Dr. Aiguader 88, 08003 Barcelona, Spain.
Tel: +34 93 3160867
Fax: +34 93 3160901
olga.valverde@upf.edu

This article is a report on results of a project that is still in preparation. Hence, although it has been included and structured as a full article, it may be significantly modified prior to being sent for peer review.

ABSTRACT

Maltreatment early in life induces pathological disturbances leading to negative emotional states. Therefore, early-life stress enhances the vulnerability to develop substance use disorders, principally during adolescence since the brain is under a maturation process. We investigated the consequences of maternal separation, a validated model of early-life stress, on drug dependence and neuroplasticity of dopaminergic system since its implication in the reward function. Our results showed that mice exposed to maternal separation displayed attenuated rewarding properties of cocaine and a decrease behavioural sensitization, whereas no changes were found in self-administration of cocaine. In addition, the evaluation of neuroplasticity in the striatal dopaminergic pathways revealed decreased protein expression levels of D2 receptors and increased of the transcriptional factor Nurr1, suggesting a hypofunctionality of the dopaminergic system accordingly with the anhedonia-like behaviour observed in mice exposed to maternal separation. Consequently, MSEW exposed to cocaine displayed increased protein expression levels of DAT, D2R and DA turnover as well as decreased of Nurr1 and Pitx3 when compared with saline-treated MSEW mice.

Keywords: Cocaine, comorbidity, early life stress, dopaminergic system

Abbreviations: Conditioned place preference (CPP); D2 dopamine receptor (D2R); Dopamine (DA); Dopamine transporter (DAT); Dihydroxyphenylacetic acid (DOPAC); Glyceraldehyde 3-phosphate dehydrogenase (GADPH); High-performance liquid chromatography (HPLC); Horseradish peroxidase (HRP); Intraperitoneal (i.p.); Intravenous (i.v.); Maternal separation with early weaning (MSEW); Nucleus accumbens (NAc); Nuclear receptor-related factor 1 (Nurr1); Postnatal day (PD); Paired-like homeodomain 3 (Pitx3), Standar nesting (SN); Tyrosine hydroxylase (TH); Vesicular monoamine transporter 2 (VMAT2); Ventral tegmental area (VTA).

1. Introduction

Adverse early life conditions in infancy have been associated with brain development alterations (Kaufman et al. 2000) increasing vulnerability to develop a psychiatric disorder throughout life (Lupien et al. 2009) such as depression (Widom et al. 2007) or substance use disorder (Gerra et al. 2009). In this term, maternal separation with early weaning (MSEW) has been proposed as an early life stress model that reproduces most of the behavioural alterations associated with mood disorders in mice at adolescence that persist in adulthood including despair, anxiety and anhedonia-like behaviour (George et al. 2010; Gracia-Rubio et al. 2015).

Consequently, adolescents with mood disorders are two to four times more likely to develop substance use disorder (Riggs 1998) suggesting that stress-induced alterations in reward processing may contribute to a higher vulnerability to addictive behaviour (Koob and Le Moal 2008). Moreover, evidence from animal studies supports the notion that acute or chronic exposure to stress increases initiation and escalation of drug abuse (Sinha 2008). Therefore, several theories propose that drugs of abuse are used in efforts to self-medicate during emotional disorders in order to relieve the feelings of sadness and anhedonia (Weiss et al. 2009). Indeed, increasing evidence in humans shows that depressive states are likely determinants of cocaine use and abuse vulnerability (Cheetham et al. 2010). Moreover, cocaine is the most used illicit drug abused after cannabis derivatives causing serious health pubic problems (WDR-UNODC, 2015).

In addition, adolescence is a critical period in which the main brain areas involved in cognitive and emotional skills are under maturation process (Crews et al. 2007). Furthermore, experimental studies have shown that during adolescence the mesocorticolimbic dopamine (DA) system, one of the most critical neural system relevant to processing salient events,

including responses to psychostimulants is undergoing developing changes (Everitt and Wolf 2002; Burke and Miczek 2014). This system is originated in the ventral tegmental area (VTA) which provides dopaminergic innervation not only to the nucleus accumbens (NAc), which is the most important substrate for reward, but also to the amygdala and the prefrontal cortex (Koob and Volkow 2010). Interestingly, several transcription factors regulate the maintenance of dopaminergic system including the orphan nuclear receptor Nurr1 and the paired-like homeobox gene, Pitx3 (Reddy et al. 2011). Nurr1 activates the transcription of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of DA, DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) (Reddy et al. 2011). Additionally, Nurr1 expression is regulated by DA signalling, principally through D_2 dopamine receptor (D2R). Consequently, D2R knockout (KO) mice have shown increased Nurr1 expression in midbrain DA neurons (Tseng et al. 2000). Pitx3 is an essential modulator of Nurr1-mediated transcription in midbrain DA and a crucial factor for specification of DA phenotype (Reddy et al. 2011). In addition, experimental studies have shown that cocaine use induces adaptive changes in cellular and synaptic function including alterations in the dopaminergic system (Leo et al. 2007) as well as stress situations (Enman et al. 2015). Several studies have tried to elucidate the link between emotional disorders and substance use disorder, nevertheless, only few studies have evaluated the effects of stress and drugs of abuse in rodents during adolescence (Doremus-Fitzwater and Spear 2010; Rodríguez-Arias et al. 2015), resulting necessary further investigation in this research area.

Hence, in this study we investigated the rewarding, addictive effects, and dopaminergic system modifications induced by cocaine in adolescent male mice exposed to MSEW. We used CD1 mice to evaluate the effects of two experimental rearing paradigms MSEW and standard nest (SN) on

the rewarding properties of cocaine by conditioned place preference (CPP), cocaine-induced locomotion and the reinforcing properties of cocaine with the self-administration procedure. In addition, we studied the cocaine-induced modifications in the dopaminergic system after CPP in order to elucidate the neuroplasticity alterations in mice exposed to early life adverse experiences.

2. Materials and methods

2.1. Animals

We used 36 male and 36 female outbred CD1adult mice as breeders purchased in Charles River, Barcelona, Spain, and shipped to our animal facility, UBIOMEX, PRBB. For the procedure of breeding, mice were housed in pairs in standard cages in a temperature- (21°± 1°C), humidity- (55% ± 10%), and light-cycle-controlled room. The room was lit between 8:00 h and 20:00 h, and experiments were conducted during the light phase (8:30 to 15:00 h) except for the self-administration procedure, in which the room was lit between 20:00-8:00 h. Food and water were available *ad libitum* for mothers and offspring except during behavioural evaluation of the offspring. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research, and were approved by the local ethics committee (CEEA-PRBB).

2.2. Rearing conditions

The rearing conditions used were conducted as previously described (Gracia-Rubio et al., 2015). Briefly, mice were randomly assigned to the different experimental groups, SN and MSEW. In the MSEW group, offspring were separated from their mothers for 4 h per day on post natal (PD) PD2-5 (9:30-13:30 h) and 8 h per day on PD6-16 (9:30-17:30 h)

(George et al., 2010; Gracia-Rubio et al., 2015). Pups from MSEW group were weaned at PD17. Pups from SN group remained with their mothers until PD21 and were then weaned (PD21). After weaning, offspring were housed in groups of 4 animals of the same sex; females were not used in the ulterior experiments.

2.3. Drug and injection procedure

Cocaine was obtained from Agencia del Medicamento (Ministery of Health, Spain) and was dissolved in sterile physiology saline (0,9%). For the conditioned place preference paradigm (CPP) experiments, cocaine was administered by intraperitoneal route (i.p.) at the doses of 1.5, 3, and 15 mg/kg. For locomotor sensitization study, cocaine was injected at the dose of 7.5 mg/kg (i.p.). For the self-administration procedure, cocaine was administered at the dose of 1mg/kg per infusion by intravenous (i.v.) route.

2.4. Conditioned Place Preference Paradigm

2.4.1 Procedure

Details of the apparatus and the procedure of cocaine CPP are described in the Supplementary material and follow the protocol described previously with minor modifications (Tourino et al. 2012).

2.4.2. Preparation of tissue extract

Mice were sacrificed by decapitation 1 h after the test. The brains were rapidly removed and stored immediately at -80ºC until use for western blot analysis (DAT, DR2, Nurr1 and Pitx3) and high performance liquid chromatography (HPLC) analysis (DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) levels). Brains were sliced on a cryostat and kept at -20ºC until each region of interest comes into the cutting plane. NAc and VTA were micro-punched from frozen brain

sections (500 µm), sectioned using a cryostat, according to the mice brain atlas of Frankin and Paxinos (2008). All micro-punched samples were stored frozen at -80ºC.

2.4.3. Electrophoresis and Western Blotting

Bilateral punches from NAc and VTA, respectively, were placed in homogenization buffer. Western blot was performed as described previously (García-Pérez et al. 2014; García-Pérez et al. 2015b). The following primary antibodies were used rabbit polyclonal anti-Nurr1 (1:500; sc-991, Santa Cruz Biotechnology, Santa Cruz, CA,USA); rabbit polyclonal anti-Pitx3 (1:750; ab30734, Abcam, Cambridge, UK); and rat monoclonal anti-DAT (1:2000; MAB369, Millipore), rabbit polyclonal anti D2R (1:500;AB5084P, Millipore). Goat anti-rabbit immunoglobulin G (IgG), Horseradish peroxidise (HRP)-linked (1:5000; sc-2004, Santa Cruz, Biotechnology) or goat anti-rat IgG, HRP-linked (1:5000;sc-2032, Santa Cruz Biotechnology) were used as secondary antibodies. Blots were incubated with stripping buffer (glycine 25mM, SDS 1%, pH2) for 1 hour at 37ºC and subsequently reblocked and probed with rabbit polyclonal antiglyceraldehyde 3-phosphate dehydrogenase (GADPH) (Cell Signaling Technology Inc., Danvers, MA, USA) or α-tubulin (Cell Signaling Technology Inc.), which were used as loading control. The ratios DAT/GADPH, D2R/GADPH, Nurr1/α-tubulin and Pitx3/α-tubulin were plotted and analysed. Proteins levels were corrected for individual levels. See Supplementary material for detail.

2.4.4. Estimation of DA and its metabolite DOPAC

DA and DOPAC were determined in the NAc by HPLC with electrochemical detection as previously described (García-Pérez et al. 2015a). See Supplementary material for detail.

2.5. Cocaine-induced locomotor sensitization procedure

The sensitization to the hyperlocomotor response elicited by cocaine (7,5 mg/kg, i.p.) was evaluated in adolescent male mice accordingly to a procedure previously described (Blanco et al., 2012) with minor modifications. Locomotor activity was measured in a low luminosity environment (20 lux) for 30 min the following days: the acute effect of cocaine was evaluated on day 3, and the effect of repeated cocaine treatment was evaluated after 5 days of cocaine treatment, on day 7. The possible effect of cocaine-conditioned locomotion during the repeated exposure to the locomotor activity boxes was evaluated after a single injection of saline, on day 13. Finally, the cocaine-induced sensitization to hyperlocomotor effects of cocaine was assessed after receiving a cocaine challenge injection (7.5 mg/kg, i.p.) on day 14.

2.6. Operant cocaine self-administration

Cocaine self-administration sessions were performed in accordance with the previously described protocols with minor modifications (Martini and Valverde 2011). Details of the apparatus, surgery and the cocaine self-administration procedure are included in the Supplementary material.

2.7. Statistical analysis

To analyse data obtained in the CPP as well as western blot and HPLC experiments a two-way ANOVA were calculated with rearing groups and treatment factors of variation, followed the Bonferroni post-hoc test. For the cocaine locomotor sensitizacion studies, we calculated a three-way ANOVA with repeated measures (day factor) and between factors (rearing groups and treatment) followed by the Bonferroni post-hoc test. To analyse data from the 10-days training in the self-administration paradigm, a three-way ANOVA was calculated with day, treatment and rearing conditions as factor of variation. Data are represented as mean ±

SEM. A p-value<0.05 was considered statistically significant. Data were analysed using SPSS v19.

3. Results

3.1. Conditioned place preference

The effect of maternal separation on the rewarding properties of cocaine (1.5; 3 and 15 mg/kg) was evaluated in adolescent male mice using the conditioned place preference paradigm. Two-way ANOVA showed a treatment effect ($F(3,83)=6,30$; $p<0,001$) without rearing group effect, nor interaction between both factors. Bonferroni post-hoc test in SN group showed a significantly effect for cocaine at doses of 3 and 15 mg/kg versus saline group ($p<0.001$) respectively. No significant differences were found in the MSEW group of mice (Fig. 1).

3.2. Expression levels of DAT and D2R proteins and DA turnover in the NAc

The effects of maternal separation on DAT, D2R and DA turnover were evaluated after performing cocaine-induced CPP. Two-way ANOVA analysis for ***DAT protein levels in the NAc*** showed a significant effect of group ($F(1,25)=5.061$; $p<0.05$), treatment ($F(2,25)=4.157$; $p<0.05$), and an interaction between these factors ($F(2,25)=5.026$; $p<0.05$). Bonferroni post-hoc analysis revealed that cocaine-treatments (3 or 15 mg/kg) significantly ($p<0.05$, $p<0.01$, respectively) increase the levels of DAT in MSEW mice when compared with the animals treated with saline (Fig. 2A). Two-way ANOVA for ***D2R protein levels*** also showed no rearing group effect ($F(1,26)=2.37$; NS), no treatment effect ($F(2,26)=1.393$; NS) but a significant interaction between group and treatment ($F(2,25)=6.502$; $p<0.05$). Bonferroni post-hoc analysis showed that MSEW group treated with saline or cocaine (3 mg/kg) displayed a decrease in the D2R levels ($p<0.05$) when compared with the SN saline-treated group. Moreover,

D2R was increased in the MSEW group treated with higher dose of cocaine (15 mg/kg) when compared with saline group ($p<0.01$). Cocaine (15 mg/kg) treatment also induced increase levels of D2R in MSEW animals when compared with the SN group treated with the same doses ($p<0.05$) (Fig. 2B).

To evaluate whether the activity of midbrain dopaminergic neurons was altered in SN and MSEW mice, after cocaine injection, DA content (data not shown), DOPAC production (data not shown) and DA turnover (as estimated by the ratio DOPAC/DA) were calculated in the NAc. Two-way ANOVA of ***DA turnover*** showed a group effect ($F(1,24)=9.53$; $p<0.01$) without treatment effect ($F(2,24)=2.65$; NS), nor interaction between these two factors ($F(2,24)=3.36$; NS). Bonferroni post-hoc test showed a significant increase in DA turnover in the MSEW group treated with higher dose of cocaine when compared with animals treated with saline ($p<0.05$). In addition, DA turnover was increased in the MSEW group treated with cocaine (15 mg/kg) when compared with SN group treated with the same dose of cocaine ($p<0.01$) (Fig. 2C).

3.3. Expression levels of Nurr1 and Pitx3 proteins on VTA

We also evaluated the expression levels of dopaminergic factors Nurr1 and Pitx3 in the VTA of mice previously exposed to the cocaine-induced CPP. Two-ANOVA for ***Nurr1 levels in the VTA*** showed no rearing group effect ($F(1,20)=0.64$; NS), significant effect of treatment ($F(2,20)=4.74$; $p<0.05$) and a significant interaction between both factors ($F(2,20)=7.09$; $p<0.01$). Bonferroni post-hoc test revealed a significant decrease in Nurr1 levels after cocaine-treatments (3 or 15 mg/kg; $p<0.05$; $p<0.01$, respectively) in the MSEW group when compared with saline-injected mice. Unexpectedly, MSEW animals receiving saline showed significant ($p<0.05$) elevation in Nurr1 levels versus the SN group (Fig.3A)

Two-way ANOVA for ***Pitx3 levels*** revealed rearing group effect ($F(1,20)=8.90$; $p<0.05$), no effect of treatment ($F(2,20)=2.76$; NS) with interaction between these two factors ($F(2,20)=3.92$; $p<0.05$). Bonferroni post-hoc analysis showed a significant ($p<0.05$) decrease in MSEW group treated with cocaine (15 mg/kg) when compared with the same group injected with saline. However, lower doses of cocaine (3 mg/kg) produced a significant increase ($p<0.05$) in Pitx3 levels in MSEW group versus SN group (Fig. 3B).

3.4. Cocaine-induced locomotor sensitization

The effect of maternal separation on locomotor sensitization induced by cocaine (7.5 mg/kg) was evaluated in male adolescent mice. No differences in the basal locomotor activity were found between rearing groups (data not shown). Three-way ANOVA with repeated measures (day factor) calculated for the locomotor activity induce by cocaine showed an effect of the rearing group, the treatment and day effect with interaction between rearing group and day, treatment and day, and between the three factors. Values for the three-way ANOVA are shown in Table 1.

Bonferroni post-hoc test showed that acute (day 3) and repeated (day 7) administration of cocaine (7.5 mg/kg) increased the locomotor activity in both SN and MSEW groups of mice respectively when compared with the saline control group ($p<0.01$).

Repeated cocaine treatment induced significant hyperlocomotor effects in mice on day 7 (sensitization to locomotor effects) only in SN group when compared with the acute treatment ($p<0.01$). Therefore, no differences were observed between acute cocaine (day 3) and repeated cocaine effects (day 7) in MSEW group, revealing that sensitization was not properly developed in mice exposed to MSEW. Hence, the hyperlocomotion induced by repeated cocaine treatment on day 7 was attenuated in mice

from MSEW comparing with mice from SN group ($p<0.01$). After five days without cocaine treatment, on day 13, mice were evaluated for the possible conditioned hyperlocomotion developed after the repeated exposure to the locomotor activity boxes during this procedure. Thus, all groups of mice received a single injection of saline and were evaluated for the locomotor effects during 30 min. Mice pre-exposed to cocaine exhibited a significant hyperlocomotor effects comparing with mice pre-exposed to saline ($p<0.01$) on day 13, independently from the rearing conditions. On day 14, all mice received a challenge of cocaine (7.5 mg/kg) and were again evaluated in the locomotor activity boxes for locomotor sensitization to cocaine. Locomotor sensitization to cocaine was developed for SN group, revealing by a significant increase of locomotor effects on days 7 and 14 comparing with acute cocaine effects observed on day 3 ($p<0.01$). Moreover, significant differences were also observed between cocaine-induced hyperlocomotion on day 7 and on day 14 ($p<0.01$). However, mice exposed to MSEW exhibited reduced effect of cocaine in locomotion. Hence, this group of mice exhibited increased in locomotion after cocaine challenge (day 14) when compared with effects observed in the same group of mice on day 3 ($p<0.01$) and when compared with effect of repeated treatment observed on day 7 ($p<0.01$). Nevertheless, a significant attenuation of the sensitization was observed when compared with those elicited by SN group of mice on day 14 ($p<0.01$), revealing a less strong locomotor sensitization in mice exposed to MSEW (Fig. 4).

3.5. Cocaine self-administration

The effects of maternal separation in adolescent mice on the reinforcing properties of cocaine were evaluated using the self-administration procedure. SN and MSEW mice were trained to self-administer cocaine at doses of 1 mg/kg per infusion during 10 days. Accordingly to previous established criteria (see Supplementary materials for details), the percentage of mice that achieved the acquisition criteria was 33% for SN group and 31% for MSEW group. Three-way repeated measures (day factor) ANOVA indicated hole effect and interaction between hole and day (Table 2).

Bonferroni post-hoc analysis revealed than SN group discriminated between the active and inactive hole from the third session (days 3 and 4, $p<0.05$; days 5 to 10, $p<0.01$), whereas MSEW group discriminated from the second session (days 2 to 10, $p<0.01$). Our results showed no significant differences between groups in cocaine self-administration (Fig. 5).

4. Discussion

Maltreatment early in life induces pathological disturbances leading to negative emotional states (Koob 2008; Lupien 2014). The individuals suffering early maltreatment are prone to consume drugs as a results of negative reinforcement mechanisms, which could results in increased reward thresholds (Koob 2008).

In our experimental conditions, MSEW seems to be an appropriate experimental model for studying the implication of early life stress on the substance use disorders since MSEW induces behavioural and neurochemical alterations that recapitulate depressive symptoms in rodents (Gracia-Rubio et al. 2015). Consequently, we evaluated the drug addictive behaviour during adolescence in mice exposed to MSEW since little is known about the effects of negative early experiment at this age,

that is an important period in which brain regions involved in emotional control and impulsivity are under maturation processes (Crews et al. 2007; Giedd 2008).

Our results in the cocaine-induced CPP paradigm indicated that mice from SN group conditioned at 3 mg/kg and 15 mg/kg, whereas MSEW mice did not show place-conditioning at any doses used, suggesting that MSEW mice would need higher doses of cocaine to experience the rewarding effects. We hypothesized that MSEW induced modifications in the DA system altering the rewarding properties of cocaine. Consequently, our results are in agreement with the anhedonia displayed by MSEW mice at adolescence in the saccharin test (Gracia-Rubio et al. 2015). In addition, previous studies have shown a decrease rewarding effects of amphetamine (Papp et al. 1991) and morphine (Valverde et al. 1997) in animals exposed to chronic mild stress. Moreover, in a neonatal model of stress has also been reported a decrease in the rewarding properties of cocaine evaluated in the CPP (Hays et al. 2012). However, a different model of maternal separation showed an enhancement in rewarding effects of morphine evaluated in the CPP (Vazquez et al. 2007), as well as in adolescent social defeat mice using cocaine (Rodríguez-Arias et al. 2015), suggesting an increase sensitivity in stressed mice.

Strikingly, acute administration of cocaine (7.5 mg/kg) induced similar enhanced locomotor activity in mice exposed to SN and MSEW groups. Although the repeated administration of cocaine increased locomotor activity in SN and MSEW groups of mice, this increase was higher in SN group, indicating reduced cocaine effect in MSEW mice. Furthermore, cocaine-pretreated SN mice showed higher locomotor activity than MSEW, whereas no differences were found between saline-pretreated groups in the challenge day. We concluded that MSEW was involved in the capability of cocaine to induce behavioural sensitization. MSEW decreases the intensity of behavioural sensitization compared with SN

group probably reducing the ability of cocaine to induce the neuroadaptations for the development of this behaviour. Additionally, the dose of cocaine selected for our study (7.5 mg/kg) was lower than those used in previous studies evaluating behaviour sensitization (Kikusui et al. 2005). Therefore, our findings evaluating sensitization in mice exposed to MSEW are in line with our results observed in the place preference paradigm that is a reduction in the cocaine effects in mice exposed to MSEW. Consequently, the lower sensitivity to the psychostimulant might lead to increase experiences with the drug to experience its pleasurable effects which is strongly linked to the initial phases for the development of addictive disorders (Planeta and Marin 2002).

Previous studies using maternal separation models showed no consistent results regarding the psychostimulants effects. Thus, an increase in cocaine sensitization after maternal separation was observed in adult mice (Matthews et al. 1999; Kikusui et al. 2005). However, other studies have shown no differences in behavioural sensitization in adolescent male rats after maternal separation were reported (Planeta and Marin 2002). Our experimental conditions were different to those reported in the previous studies for both, the maternal separation and the sensitization procedures, and consequently, clear conclusions are difficult to be drawn.

We have also evaluated cocaine-induced reinforcing effects in the i.v. self-administration. In this study, no differences between rearing conditions were found probably due to the dose of cocaine (1 mg/kg per infusion) selected and the duration of the training period. Therefore, further studies are required to complete this study. In this sense, preclinical studies have found that early life stress can modulate the rewarding mesolimbic circuits in adulthood; both increasing reward induced behavioural-reinforcement (Matthews et al. 1999; Kosten et al. 2003) or reducing rewarding responses, probably due to the development of anhedonia (Martini and Valverde 2011; O'Connor et al. 2015). Other

studies evaluating different models of repeated stress such as social defeat have also shown controversial data. Consequently, whereas adult social defeat stress resulted in an escalated cocaine self-administration in socially stressed adult mice (Han et al. 2015), no differences were found in social stressed adolescent mice (Rodríguez-Arias et al. 2015).

Regarding the molecular evaluation of the dopaminergic system in mice exposed to MSEW, a decrease in D2R protein expression levels in the NAc and an increase of the transcriptional factors Nurr1 in the VTA during adolescence were found. However, no differences in DAT, DA turnover or the transcriptional factor Pitx3 were observed. The decrease in D2R in saline-treated MSEW mice could reflect the anhedonia-like effects observed in behavioural studies, and the attenuation of rewarding activity of mesolimbic system. Accordingly, a model of PTSD displayed a decrease in D2R in NAc and showed anhedonia-like behaviour as well as a decrease in cocaine intake in self-administration procedure (Enman et al. 2015). In agreement, mice exposed to stressful situations during several days also showed a D2R density reduction in the NAc (Puglisi-Allegra et al. 1991). At this point, we must consider the hypothesis by Volkow and colleagues, proposing that high levels of D2R confer resilience to developed substance use disorders whereas low levels of D2R increase vulnerability to suffer this psychiatric disorder (Volkow et al. 2002). This hypothesis supports our results regarding the lack of reward observed in conditioned place preference, since MSEW mice probably required high doses of cocaine to experience reward. Accordingly, the administration of D2R antagonist, sulpiride (100 mg/kg, i.p.), reduced cocaine sensitization (Mattingly et al., 1994) and blocked cocaine, morphine and alcohol rewarding effects in the place preference paradigm (Matsuzawa et al. 1999; Rezayof et al. 2002; Nazarian et al. 2004). Interestingly, knockout mice lacking D2R receptors exhibited a higher cocaine self-administration than wild-type littermates only at higher doses of cocaine, whereas no

differences were found between genotypes when using lower doses of cocaine (0.03 and 3.2 mg/kg per infusion) (Caine et al. 2002). Other studies have also evaluated the participation of maternal separation on the dopaminergic function, reporting no differences in D2R density in adult mice (Vazquez et al. 2007) while a model of prenatal stress found an increase in the expression of these dopaminergic receptors (Katunar et al. 2009). These results are in line with cocaine sensitization results here reported. Therefore, the decrease of D2 receptors could be related to the reduction of cocaine-induced locomotion in mice exposed to MSEW. In addition, our data also show the existence of a compensatory mechanism to enhance the impaired dopaminergic activity through an increased expression of Nurr1 protein levels in the VTA. Nurr1 is a transcription factor essential for the differentiation of midbrain DA neurons (Jankovic et al. 2005). Therefore, D2R KO mice exhibited an enhancement of Nurr1 levels in the VTA and substance nigra whereas no differences were found in other brain regions such as cortex or habenular nuclei (Tseng et al. 2000).

Our results in SN mice during cocaine-induced conditioned place preference showed no changes in protein expression of DAT, D2R, Nurr1, and Pitx3 as well as in DA turnover, suggesting that cocaine did not produce neuroadaptations in the dopaminergic system after a sub-chronic cocaine treatment. It is important to note that mice were sacrificed 48 h after the last cocaine injection when no expected residual psychostimulant effects remained. In this sense, decreases in Nurr1 and Pitx3 protein were found after 14 days of cocaine treatment, without changes after acute or shorter cocaine treatment. Surprisingly, no differences were observed in TH, DAT, VMAT2 or D2R after 14 days of cocaine treatment (Leo et al. 2007). In those conditions, the decreased expression of Nurr1 and Pitx3 can be viewed as a homeostatic response to increased DA neurotransmission after the cocaine treatment. Moreover, the lack of

modulation of other genes affecting DA neurotransmission after chronic cocaine administration suggests that the partial down-regulation of Nurr1 and Pitx3 may not be enough to modify the expression of some putative downstream target genes (Leo et al. 2007). However, the morphine (acute and chronic treatments) and the morphine withdrawal enhances the expression of the former dopaminergic factors inducing alterations in the expression of DAT, VMAT and D2R (García-Pérez et al. 2014; García-Pérez et al. 2015b). Our results lead us to suggest that mice exposed to MSEW developed a hypofunction of the dopaminergic system as revealed in saline-treated mice. This dopaminergic dysfunction is hypothesized to contribute to drug-seeking and drug-taking behaviours (Morgan et al. 2002; Koob and Volkow 2010). Therefore, the intermittent administration of cocaine in MSEW mice was able to induce neuroadaptations in the dopaminergic system, which would be involved in compensatory mechanisms. Consequently, MSEW exposed to cocaine displayed increased protein expression levels of DAT, D2R and DA turnover as well as decreased of Nurr1 and Pitx3 when compared with saline-treated MSEW mice. However, further experiments would be needed to clarify the implication of MSEW model in the acute and long-term regulation of the dopaminergic system. Although our results showed an attenuation of cocaine rewarding effects and cocaine-induced sensitization, we conclude that MSEW is an appropriate model to investigate the influence of anhedonia (a main symptom of depression) on substance use disorders, due to the higher vulnerability to develop addictive disorders presumably related to the reduction in dopaminergic activity in circuits controlling reward.

ACKNOWLEDGEMENTS

This study was supported by MINECO (SAF2013-41761-R-FEDER), Ministry of Health (Retic-ISCIII-RD/12/0028/0024-FEDER and *Plan Nacional sobre Drogas* 2014/020), and *Generalitat de Catalunya* (2014SGR34). IG-R was funded by FPI fellowship BES-2011-046655 associated with SAF2010-15793.

REFERENCES

Burke AR, Miczek KA (2014) Stress in adolescence and drugs of abuse in rodent models: role of dopamine, CRF, and HPA axis. Psychopharmacology (Berl) 231:1557–80. doi: 10.1007/s00213-013-3369-1

Caine SB, Negus SS, Mello NK, et al (2002) Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. J Neurosci 22:2977–88. doi: 20026264

Cheetham A, Allen NB, Yücel M, Lubman DI (2010) The role of affective dysregulation in drug addiction. Clin Psychol Rev 30:621–34. doi: 10.1016/j.cpr.2010.04.005

Crews F, He J, Hodge C (2007) Adolescent cortical development: a critical period of vulnerability for addiction. Pharmacol Biochem Behav 86:189–99. doi: 10.1016/j.pbb.2006.12.001

Doremus-Fitzwater TL, Spear LP (2010) Age-related differences in amphetamine sensitization: effects of prior drug or stress history on stimulant sensitization in juvenile and adult rats. Pharmacol Biochem Behav 96:198–205. doi: 10.1016/j.pbb.2010.05.005

Enman NM, Arthur K, Ward SJ, et al (2015) Anhedonia, Reduced Cocaine Reward, and Dopamine Dysfunction in a Rat Model of Posttraumatic Stress Disorder. Biol Psychiatry. doi: 10.1016/j.biopsych.2015.04.024

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22:3312–20. doi: 20026356

García-Pérez D, López-Bellido R, Hidalgo JM, et al (2015a) Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. Addict Biol 20:104–19. doi: 10.1111/adb.12083

García-Pérez D, López-Bellido R, Rodríguez RE, et al (2015b) Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. Brain Struct Funct 220:1901–19. doi: 10.1007/s00429-014-0761-5

García-Pérez D, Núñez C, Laorden ML, Milanés MV (2014) Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. Addict Biol. doi: 10.1111/adb.12209

George ED, Bordner KA, Elwafi HM, Simen AA (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

Gerra G, Leonardi C, Cortese E, et al (2009) Childhood neglect and parental care perception in cocaine addicts: relation with psychiatric symptoms and biological correlates. Neurosci Biobehav Rev 33:601–10. doi: 10.1016/j.neubiorev.2007.08.002

Giedd JN (2008) The teen brain: insights from neuroimaging. J Adolesc Health 42:335–43. doi: 10.1016/j.jadohealth.2008.01.007

Gracia-Rubio I, Moscoso-Castro M, Pozo OJ, et al (2015) 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

Han X, Albrechet-Souza L, Doyle MR, et al (2015) Social stress and escalated drug self-administration in mice II. Cocaine and dopamine in the nucleus accumbens. Psychopharmacology (Berl) 232:1003–10. doi: 10.1007/s00213-014-3734-8

Hays SL, McPherson RJ, Juul SE, et al (2012) Long-term effects of neonatal stress on adult conditioned place preference (CPP) and hippocampal neurogenesis. Behav Brain Res 227:7–11. doi: 10.1016/j.bbr.2011.10.033

Jankovic J, Chen S, Le WD (2005) The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. Prog Neurobiol 77:128–38. doi: 10.1016/j.pneurobio.2005.09.001

Katunar MR, Saez T, Brusco A, Antonelli MC (2009) Immunocytochemical expression of dopamine-related transcription factors Pitx3 and Nurr1 in prenatally stressed adult rats. J Neurosci Res 87:1014–22. doi: 10.1002/jnr.21911

Kaufman J, Plotsky PM, Nemeroff CB, Charney DS (2000) Effects of early adverse experiences on brain structure and function: clinical implications. Biol Psychiatry 48:778–790. doi: 10.1016/S0006-3223(00)00998-7

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. doi: 10.1007/s00213-004-1989-1

Koob GF (2008) A role for brain stress systems in addiction. Neuron 59:11–34. doi: 10.1016/j.neuron.2008.06.012

Koob GF, Le Moal M (2008) Review. Neurobiological mechanisms for

opponent motivational processes in addiction. Philos Trans R Soc Lond B Biol Sci 363:3113–23. doi: 10.1098/rstb.2008.0094

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217–238. doi: 10.1038/npp.2010.4

Kosten TA, Zhang XY, Kehoe P (2003) Chronic neonatal isolation stress enhances cocaine-induced increases in ventral striatal dopamine levels in rat pups. Brain Res Dev Brain Res 141:109–16.

Leo D, di Porzio U, Racagni G, et al (2007) Chronic cocaine administration modulates the expression of transcription factors involved in midbrain dopaminergic neuron function. Exp Neurol 203:472–80. doi: 10.1016/j.expneurol.2006.08.024

Lupien SJ (2014) The double-hit effect of childhood maltreatment on drug relapse. JAMA psychiatry 71:871–2. doi: 10.1001/jamapsychiatry.2014.924

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445. doi: 10.1038/nrn2639

Martini M, Valverde O (2011) A single episode of maternal deprivation impairs the motivation for cocaine in adolescent mice. Psychopharmacology (Berl) 219:149–158. doi: 10.1007/s00213-011-2385-2

Matsuzawa S, Suzuki T, Misawa M, Nagase H (1999) Involvement of dopamine D(1) and D(2) receptors in the ethanol-associated place preference in rats exposed to conditioned fear stress. Brain Res 835:298–305.

Matthews K, Robbins TW, Everitt BJ, Caine SB (1999) Repeated neonatal maternal separation alters intravenous cocaine self-administration in adult rats. Psychopharmacology (Berl) 141:123–34.

Morgan D, Grant KA, Gage HD, et al (2002) Social dominance in monkeys: dopamine D2 receptors and cocaine self-administration. Nat Neurosci 5:169–74. doi: 10.1038/nn798

Nazarian A, Russo SJ, Festa ED, et al (2004) The role of D1 and D2 receptors in the cocaine conditioned place preference of male and female rats. Brain Res Bull 63:295–9. doi: 10.1016/j.brainresbull.2004.03.004

O'Connor RM, Moloney RD, Glennon J, et al (2015) Enhancing glutamatergic transmission during adolescence reverses early-life stress-induced deficits in the rewarding effects of cocaine in rats. Neuropharmacology 99:168–176. doi: 10.1016/j.neuropharm.2015.07.012

Ojeda V, Fuentealba JA, Galleguillos D, Andrés ME (2003) Rapid increase of Nurr1 expression in the substantia nigra after 6-hydroxydopamine lesion in the striatum of the rat. J Neurosci Res 73:686–97. doi: 10.1002/jnr.10705

Papp M, Willner P, Muscat R (1991) An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. Psychopharmacology (Berl) 104:255–9.

Planeta CS, Marin MT (2002) Effect of cocaine on periadolescent rats with or without early maternal separation. Braz J Med Biol Res 35:1367–71.

Puglisi-Allegra S, Kempf E, Schleef C, Cabib S (1991) Repeated stressful experiences differently affect brain dopamine receptor subtypes. Life Sci 48:1263–8.

Reddy SDN, Rayala SK, Ohshiro K, et al (2011) Multiple coregulatory control of tyrosine hydroxylase gene transcription. Proc Natl Acad Sci U S A 108:4200–5. doi: 10.1073/pnas.1101193108

Rezayof A, Zarrindast M-R, Sahraei H, Haeri-Rohani A-H-R (2002) Involvement of dopamine D2 receptors of the central amygdala on the acquisition and expression of morphine-induced place preference in rat. Pharmacol Biochem Behav 74:187–97.

Riggs PD (1998) Clinical approach to treatment of ADHD in adolescents with substance use disorders and conduct disorder. J Am Acad Child Adolesc Psychiatry 37:331–2.

Rodríguez-Arias M, Montagud-Romero S, Rubio-Araiz A, et al (2015) 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. doi: 10.1111/adb.12301

Sinha R (2008) Chronic stress, drug use, and vulnerability to addiction. Ann N Y Acad Sci 1141:105–30. doi: 10.1196/annals.1441.030

Tourino C, Valjent E, Ruiz-Medina J, et al (2012) The orphan receptor GPR3 modulates the early phases of cocaine reinforcement. Br J Pharmacol 167:892–904. doi: 10.1111/j.1476-5381.2012.02043.x

Tseng KY, Roubert C, Do L, et al (2000) Selective increase of Nurr1 mRNA expression in mesencephalic dopaminergic neurons of D2 dopamine receptor-deficient mice. Brain Res Mol Brain Res 80:1–6.

Valverde O, Smadja C, Roques BP, Maldonado R (1997) The attenuation of morphine-conditioned place preference following chronic mild stress is reversed by a CCKB receptor antagonist. Psychopharmacology (Berl) 131:79–85.

Vazquez V, Weiss S, Giros B, et al (2007) Maternal deprivation and handling modify the effect of the dopamine D3 receptor agonist, BP 897 on morphine-conditioned place preference in rats. Psychopharmacology (Berl) 193:475–86. doi: 10.1007/s00213-007-0789-9

Volkow ND, Wang G-J, Fowler JS, et al (2002) Brain DA D2 receptors predict reinforcing effects of stimulants in humans: replication study. Synapse 46:79–82. doi: 10.1002/syn.10137

Weiss RD, Griffin ML, Mirin SM (2009) Drug Abuse as Self-Medication for Depression: An Empirical Study.

Widom CS, DuMont K, Czaja SJ (2007) A prospective investigation of major depressive disorder and comorbidity in abused and neglected children grown up. Arch Gen Psychiatry 64:49–56. doi: 10.1001/archpsyc.64.1.49

LEGENDS OF FIGURES

Figure 1. Effects of maternal separation on the rewarding properties of cocaine (1.5, 3 and 15 mg/kg) in the conditioned place preference paradigm. Data are expressed as the mean (± SEM) of the score calculated in the CPP (see Supplementary materials for details). N=8–15 mice per group. **p<0.01 vs. saline group.

Figure 2. Effect of maternal separation on DAT, D2R levels and DA turnover in the NAc. (**A,B**) Densitometric analysis of specific integrated optical density (% of control) signals normalized to the corresponding GADPH levels and representative Western-blot analysis of DAT and D2R in the NAc micropunches, respectively. (**C)** DA turnover (as determined by the DOPAC/DA ratio) in the NAc. Each bar corresponds to mean ± SEM, N=4-6 mice per group. * p<0.05, ** p<0.01 vs. MSEW group treated with saline; + p<0.05 vs. SN injected with saline; & p<0.05 vs. SN treated with cocaine (3 mg/kg); $ p<0.05 vs. SN group treated with cocaine (15 mg/kg).

Figure 3. Effects of maternal separation on Nurr1 and Pitx3 levels in the VTA. Densitometric analysis of specific integrated optical density (% of control) signals normalized to the corresponding α-tubulin levels and representative Western-blot analysis of Nurr1 (A) and Pitx3 (B) levels in the VTA michopunches. Each bar corresponds to mean ± SEM, N=4-5 mice per group. * p<0.05, ** p<0.01 vs. MSEW group treated with saline; + p<0.05 vs. SN injected with saline; & p<0.05 vs. SN treated with cocaine (3 mg/kg).

Figure 4. Effects of maternal separation on the cocaine locomotor sensitization. Data are expressed as the mean (± SEM) of cumulative breaks/animal in locomotor activity boxes. N=12 mice per group. $$ p<0.01 treatment effect vs. saline on the same days. ## p<0.01, pre-treated

with cocaine vs. pre-treated with saline when conditioned hyperlocomotion was evaluated on day 13. && $p<0.01$ pre-treated with cocaine vs. pre-treated with saline when cocaine-induced sensitization was evaluated. ** $p<0.01$ rearing group effect. Two white starts $p<0.01$, day effect as indicated by the arrows.

Figure 5. Effects of maternal separation on the reinforcing properties of cocaine (1mg/kg per infusion) in the self-administration procedure. Data are expressed as the mean (± SEM) of nose-poke in the active/inactive hole in the training session along the 10 days (1 h). N=9-10 mice per group. White symbols with continued line represent nose-pokes in the active hole in SN group whereas dotted line represents nose-pokes in the inactive hole. Black symbols with continued line represents nose-pokes in the active hole in MSEW group, dotted line represents nose-pokes in the inactive hole. * $p<0.05$; ** $p<0.01$ nose-pokes in the active hole vs. inactive hole in SN group. ## $p<0.01$ nose-pokes in the active hole vs. inactive hole in MSEW group.

Table 1. Three-way ANOVA calculated to evaluate the effect of maternal separation on cocaine-induced locomotor sensitization. Rearing group (R), Treatment (T), Day (D).

	Cocaine-induced locomotor sensitization	
	F	**P <**
R	F(1,44)=4,722	0,05
T	F(1,44)=57,53	0,01
D	F(3,132)=116,8	0.01
R X T	F(1,44)=0,872	NS
R X D	F(3,132)=3,354	0.05
T X D	F(3,132)=8,521	0.01
R X T X D	F(3,132)=4,340	0,01

Table 2. Three-way ANOVA calculated for evaluating the effect of maternal separation on cocaine-self-administration. Rearing group (R), Hole (H), Day (D).

	Cocaine self-administration	
	F	**P<**
R	F(1,17)=4,722	NS
H	F(1,17)=57,53	0,01
D	F(9,153)=116,8	NS
R X H	F(1,17)=0,872	NS
R X D	F(9,153)=3,354	NS
H X D	F(9,153)=8,521	0.01
R X H X D	F(9,153)=4,340	NS

Figure 1

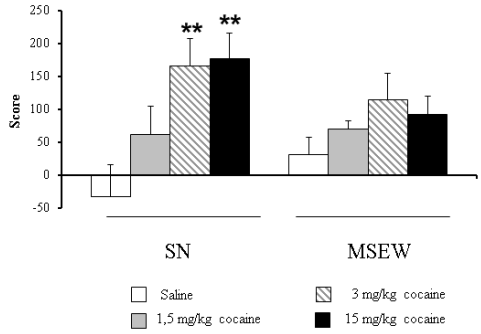


Figure 2

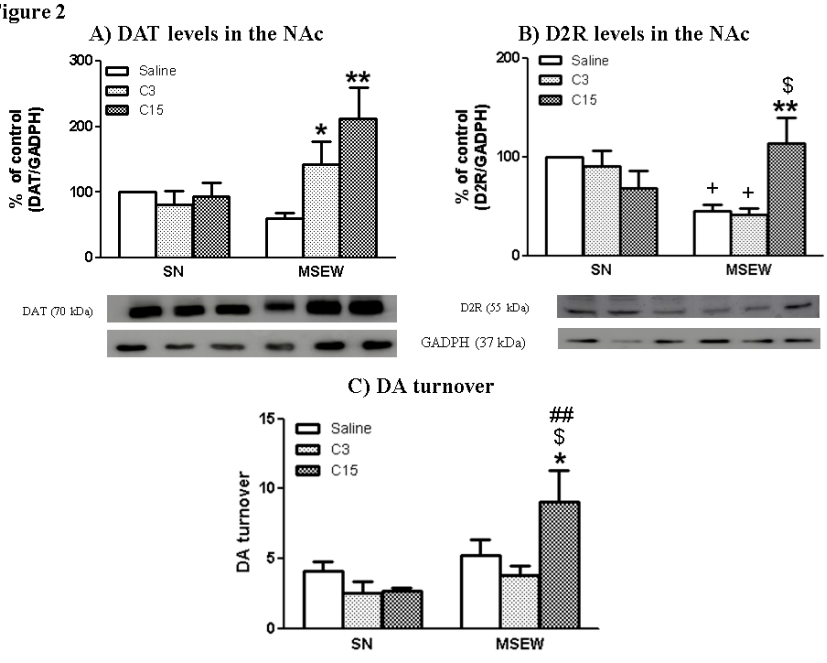


Figure 3

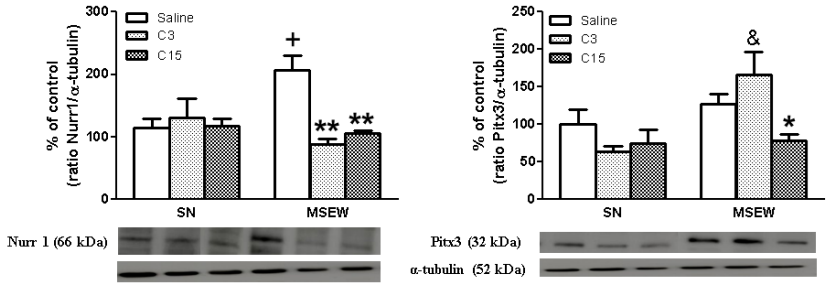


Figure 4

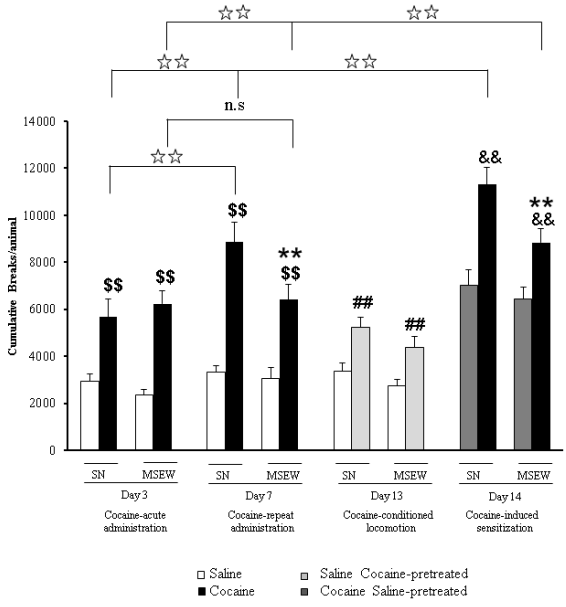
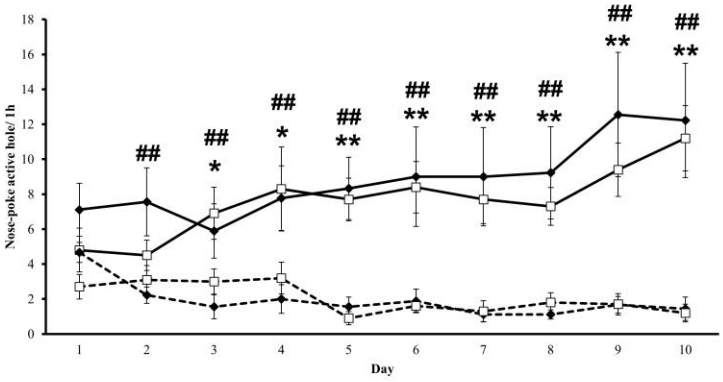


Figure 5



SUPPLEMENTARY MATERIALS

2.4. Conditioned Place Preference Paradigm

2.4.1 Procedure

In adolescent male mice, the rewarding properties of cocaine (1.5, 3 and 15 mg/kg, i.p.) were evaluated using the CPP paradigm, as previously described (Tourino et al. 2012). The conditioned placed preference was carried out in a box divided in two different compartments consisting in two square chambers (20cm x 20cm x 20cm) connected by a rectangular grey compartment (20cm x 20cm x 20cm). The two compartments differed in colours and floor textures. One of the compartments was black with a black rugous textured floor and the other compartment was white with a transparent plastic floor with black horizontal lines. A grey neutral section separated the two chambers. The procedure was conducted in three different phases, as follow:

Pre-conditioning Phase. The first phase consisted of 20 min of free exploration of the box. For that, each mouse was placed in the middle of the neutral area and was allowed to explore the both compartments. Mice showing preference or aversion for one of the compartments (more than 70% or minus than 30%) were excluded from the experiment.

Conditioning Phase. For the next six days, the mice were injected with cocaine or saline in alternate days. Mice received cocaine on days 1, 3 and 5 and received saline on days 2, 4 and 6 of the Conditioning Phase. Control animals received saline every day. In all the cases, immediately after the injection, each mouse was immediately placed into the assigned compartment during 30 min.

Testing Phase. On the eighth day, mice were allowed to explore both compartments for 20 minutes, similar to the pre-conditioning phase. The time spent in each compartment was calculated. A score was calculated as

the difference of time spent in the compartment associated to the drug on the post-conditioning and the pre-conditioning.

2.4.2. Preparation of tissue extract

Mice were sacrificed by decapitation 1 h after the test. The brains were rapidly removed and stored immediately at -80ºC until use for western blot analysis (DAT, DR2, Nurr1 and Pitx3) and high performance liquid chromatography (HPLC) analysis (DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) levels). Brains were sliced on a cryostat and kept at -20ºC until each region of interest comes into the cutting plane. NAc and VTA were micro-punched from frozen brain sections (500 µm), sectioned using a cryostat, according to the mice brain atlas of Frankin and Paxinos (2008). All micro-punched samples were stored frozen at -80ºC.

2.4.3. Electrophoresis and Western Blotting

Western blot was performed as described previously (García-Pérez et al. 2014; García-Pérez et al. 2015b). Bilateral punches from NAc and VTA were placed in a buffer containing phosphate buffered saline, 10% sodium dodecyl sulfate (SDS), protease inhibitors (Boehringer Mannheim, Mannheim, Germany) and a phosphatase inhibitor Cocktail Set (Calbiochem, Darmstadt, Germany), homogenized and sonicated for 30 s before centrifugation at 6.000g for 10 min at 4°C. Samples containing 20 µg of protein were loaded on a 10% SDS/polyacrylamide gel, electrophoresed and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Nonspecific binding of antibodies was prevented by incubating the membranes in 1% bovine serum albumin (BSA) in Tris-buffered saline Tween-20 (TBST; 10 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.15% Tween 20). The blots were incubated at 4ºC overnight with the following primary antibodies: rabbit

polyclonal anti-Nurr1 (1:500; sc-991, Santa Cruz Biotechnology, Santa Cruz, CA,USA); rabbit polyclonal anti-Pitx3 (1:750; ab30734, Abcam, Cambridge, UK); and rat monoclonal anti-DAT (1:2000; MAB369, Millipore), rabbit polyclonal anti D2R (1:500;AB5084P, Millipore). Goat anti-rabbit immunoglobulin G (IgG), Horseradish peroxidise (HRP)-linked (1:5000; sc-2004, Santa Cruz, Biotechnology) or goat anti-rat IgG, HRP-linked (1:5000;sc-2032, Santa Cruz Biotechnology) were used as secondary antibodies. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECL Plus, GE Healthcare, Little Chalfont, Buckinghamshire, UK9 and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Blots were incubated with stripping buffer (glycine 25mM, SDS 1%, pH2) for 1 hour at 37ºC and subsequently reblocked and probed with rabbit polyclonal antiglyceraldehyde 3-phosphate dehydrogenase (GADPH) (Cell Signaling Technology Inc., Danvers, MA, USA or α-tubulin (Cell Signaling Technology Inc.), which were used as loading control. The ratios DAT/GADPH, D2R/GADPH, Nurr1/α-tubulin and Pitx3/α-tubulin were plotted and analysed. Proteins levels were corrected for individual levels.

2.4.4. Estimation of DA and its metabolite DOPAC

DA and DOPAC were determined in the NAc by HPLC with electrochemical detection as previously described by (García-Pérez et al. 2015a). One punch from each animal was obtained and added to 60 µl of a solution composed by 1M $HClO_4$ (Sigma Chemical Co) and 2.7 mM ethylenediaminete-traacetic acid (Sigma Chemical Co). The samples were homogenized by slight sonication for about 1 min, centrifuged (6000xg; 4ºC) for 10 min and the supernatants were taken for analysis and filtered through ultra-free MC 0.2 µm filter (Millipore). The pellets were resuspended by adding 100 µl of 1N NaOH. Then, the total amount of

proteins from each sample was measured by spectrophotometry. From each sample 10 µl was injected into a 5-µm C18 reversed-phase column (Waters, Milford, MA, USA) through a Rheodyne syringe-loading injector 200 µL loop (Waters). The mobile phase consisted of a 95% (v/v) mixture of water and methanol with sodium acetate (50 mM) (Sigma Chemical Co), citric acid (20 mM) (Sigma Chemical Co), L-octyl-sodium sulfonate (3.75 mM) (Sigma Chemical Co), di-n-butylamine (1 mM) (Sigma Chemical Co) and EDTA (0.135 mM) (Sigma Chemical Co), adjusted to pH 4.3. Chromatographic data were analysed with Millenium 2010 Chromatography Manager Equipment (Millipore). DA and DOPAC was simultaneously detected and quantified by reference to calibration curves run at the beginning of the assays. The content of DA and DOPAC is expressed as ng/mg of protein. The DA turnover was determined as the DOPAC/DA ratio. The DOPAC/DA ratio was used as indices of transmitter metabolism.

2.5. Cocaine-induced locomotor sensitization procedure

The sensitization to the hyperlocomotor response elicited by cocaine was evaluated in adolescent male mice accordingly to a procedure previously described (Blanco et al., 2012) with minor modifications (Fig. X). Locomotor activity boxes (24 x 24 x 24 cm) (LE8811 IR, Panlab S.L., Barcelona, Spain) were used in a low luminosity room (20 lux) with white noise.

The first day, mice were handled and weighted. The following day, mice were injected with saline and their basal locomotor activities were recorded during 30 min. During the following five days, mice were daily injected with cocaine (7,5mg/kg) or saline, and then immediately placed in the locomotor activity boxes to record the locomotor activity during 30 min. After this repeated treatment with cocaine, mice remained in their home cage during five days without receiving any treatment. After this

period of time, on day 13, mice received a saline injection and the possible conditioned response associated to the environment into the locomotor activity box was evaluated. On day 14, mice received a cocaine challenge (7,5 mg/kg, ip) and then, behavior sensitization was investigated. However, only the first 15 min were analyzed.

2.6. Operant cocaine self-administration

2.6.1. Surgery for the i.v. catheter implantation

At adolescence, mice were anesthetized with ketamine/xylazine (100mg/kg, 20mg/kg respectively) and then implanted with indwelling i.v silastic catheter as described previously (Martini and Valverde 2011). Briefly, a 4.5 cm length of silastic tubing (0.3 mm inner diameter, 0.64 mm outer diameter) (SilasticR, Dow Corning, Houdeng-Goegnies, Belgium) was adapted to a 22-gauge steel cannula (Semat; Herts; England) that was curved at a 90° angle and then placed in a cement disk (Dentalon plus, Heraeus Kulzer, Germany). The catheter tubing was inserted 1.4 cm into the right jugular vein (limited with a little dab of silicone) and held with suture. The rest of the catheter passed subcutaneously (s.c) from the insertion position to the cannula that was fixed in the back of mice, this incision was closed with staples. All incisions were coated with Betadine (Meda Pharma SAU, S.Fernando de Henares, Madrid, Spain). Mice were treated with analgesia (meloxicam; 0.5mg/kg; s.c; Inflacam, Chanelle Ltd, Loughrea, Co., Galway, IE) and antibiotic (Enrofloxacino; 7.5 mg/kg; i.p; Batryl, Bayer Hispania S.L, Sant Joan Despí, Barcelona ,Spain) in order to improve the mice's recovery. After surgery, mice were allowed to recover for 3-5 days prior to start the self-administration procedure.

2.6.2. Apparatus for self-administration experiments

The self-administration experiments were carried out in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Georgia, VT, USA) containing two holes; one was defined as active and the other as inactive. Nose-poking on the active hole produced a cocaine infusion (reinforce) that was paired with two stimulus light, one placed inside the nose-poke and the other above the active hole. No consequences had nose-poking in the inactive hole. The side, in which active/inactive hole was placed, were counterbalance. The chambers were housed in sound- and-light- attenuated boxes provided with fans to provide ventilation and white noise (Fig. 16).

2.6.3. Drug self-administration procedure

Cocaine self-administration session was performed as described previously (Martini and Valverde 2011) with minor modifications. Responding was maintained by cocaine (1mg/kg per infusion) delivered in 20µl over 2s. Cocaine was infusion via a syringe that was set on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) and 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 to the mouse i.v. catheter. Self-administration sessions (1 h daily) were conducted during 10 consecutive days. At the beginning the house light was on during 3 seconds and off during the rest of the session. The session started with a priming infusion of the drug. Mice were trained under a fixed ratio 1 (FR1) schedule of reinforcement. The number of reinforces was limited to 50 per session to avoid overdoses and each reinforce was followed by a 30 s time-out period where nose-poking in the active hole had no consequences. A mouse was considered to acquire when the number of responses in the active hole was at least 5, exceeded 75% of

that on the inactive hole and it maintained a stable responding with less than 30% deviation from the mean of the total number of cocaine infusions obtained in two consecutive days (70% of stability). The patency of the intravenous catheters was evaluated at the end of the experiment 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 anaesthesia were not appear within 3 seconds, the mouse was removed from the experiment.

Article 3

Addiction Biology

Addiction Biology SSA SOCIETY FOR THE STUDY OF ADDICTION

Ethanol binge drinking enhances cocaine-induced sensitization and reinforcement in adolescent mice

Journal:	*Addiction Biology*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Esteve-Arenys, Anna; Grup de Recerca en Neurobiologia del Comportament (GReNeC). , Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra Gracia-Rubio, Irene; Grup de Recerca en Neurobiologia del Comportament (GReNeC), Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra. Cantacorps, Lídia; Grup de Recerca en Neurobiologia del Comportament (GReNeC), Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra Pozo, Oscar; Bioanalysis Research Group IMIM-Hospital del Mar Medical Research Institute Marcos, Josep; Bioanalysis Research Group, IMIM-Hospital del mar Medical Research Institute Rodriguez-Arias, Marta; University of Valencia, Psychobiology Miñarro, Jose; University of Valencia, Psychobiology Valverde, Olga; Grup de Recerca en Neurobiologia del Comportament (GReNeC)., Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra.
Keywords:	Binge ethanol drinking, Cocaine, Adolescent mice
Abstract:	Binge drinking is an emerging pattern of alcohol intake in adolescents and young adults. This pattern of consumption has negatives consequences for health, and in addition to its neuropsychiatric effects, binge drinking during adolescence may aggravate the deleterious consequences of drug-taking behaviour in the adulthood. In this study, we investigated in C57BL/6J mice the effects of voluntary oral ethanol consumption during adolescence on the effects of cocaine during adulthood. We evaluated four regimes of oral ethanol intake in adolescent mice that differed in pattern of ethanol exposure (continuous versus binge), and ethanol concentration (20 % versus 30 %, v/v). We then selected the regime that resulted in highest ethanol blood concentrations (binge drinking, 20% v/v ethanol; regime B20) for further studies on the subsequent acute and long-term effects of cocaine in adulthood. Our results show that mice that exhibit a binge consumption pattern during adolescence showed higher responses to acute and repeated administration of cocaine, including increased behavioural sensitization, and an enhanced operant response to self-administered cocaine. Our findings highlight the long-lasting detrimental effects of ethanol binge drinking during adolescence, which may induce a "sensitized

Article 4

Elsevier Editorial System(tm) for European Neuropsychopharmacology

Manuscript Draft

Manuscript Number:

Title: Genetic blockade of adenosine A2A receptors induces cognitive impairments and anatomical changes related to psychotic symptoms in mice.

Article Type: Research article

Section/Category: BS - Basic Science

Keywords: Adenosine A2A
schizophrenia
cognition
psychotic behavior
ventricles enlargement
BDNF

Corresponding Author: Prof. OLGA VALVERDE,

Corresponding Author's Institution: Universitat Pompeu Fabra

First Author: Maria Moscoso-Castro

Order of Authors: Maria Moscoso-Castro; Irene Gracia-Rubio; Francisco Ciruela; OLGA VALVERDE

Abstract: Schizophrenia is a chronic severe mental disorder with a presumed neurodevelopmental origin, and no effective treatment. Schizophrenia is a multifactorial disease with genetic, environmental and neurochemical etiology. The main theories on the pathophysiological basis of this disorder include alterations in dopaminergic and glutamatergic neurotransmission in limbic and cortical areas of the brain. Early hypotheses also suggested that nucleoside adenosine is a putative affected neurotransmitter system, and clinical evidence suggests that adenosine adjuvants improve treatment outcomes, especially in poorly responsive patients. Hence, it is important to elucidate the role of the neuromodulator adenosine in the pathophysiology of schizophrenia. A2A adenosine receptor (A2AR) subtypes are highly expressed in brain areas controlling motivational responses and cognition, including striatum, hippocampus and cerebral cortex. The aim of this study was to characterize A2AR knockout (KO) mice with complete and specific inactivation of A2AR, as an animal model for schizophrenia. We performed diverse behavioral, anatomical and neurochemical studies to assess psychotic-like symptoms in adult male and female KO and wild-type (WT) littermates. Our results show overall impairment in inhibitory responses and sensory gating in A2AR KO animals. Hyperlocomotion induced by D-amphetamine and MK-801 was reduced in KO animals when compared to WT littermates. Moreover, A2AR KO animals show motor disturbances, social and cognitive alterations. Finally, behavioral impairments were associated with enlargement of brain lateral ventricles and decreased BDNF levels in the hippocampus. These data highlight the role of adenosine in the pathophysiology of schizophrenia and provide new possibilities for the therapeutic management of this mental disorder.

Moscoso-Castro M, Gracia-Rubio I, Ciruela F, Valverde O. Genetic blockade of adenosine A(2A) receptors induces cognitive impairments and anatomical changes related to psychotic symptoms in mice. Eur Neuropsychopharmacol. 2016 Apr 28. pii: S0924-977X(16)30028-1. doi: 10.1016/j.euroneuro.2016.04.003

Article 5

Targeting Tryptophan and Tyrosine Metabolism by Liquid Chromatography Tandem Mass Spectrometry.

Marcos J, Renau N, Valverde O, Aznar-Laín G, Gracia-Rubio I, Gonzalez-Sepulveda M, Pérez-Jurado LA, Ventura R, Segura J, Pozo OJ

Journal of Chromatography A

doi: 10.1016/j.chroma.2016.01.023

Marcos J, Renau N, Valverde O, Aznar-Laín G, Gracia-Rubio I, Gonzalez-Sepulveda M, Pérez-Jurado LA, Ventura R, Segura J, Pozo OJ. Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry. J Chromatogr A. 2016 Feb 19;1434:91-101. doi: 10.1016/j.chroma.2016.01.023

Article 6

Modulation of cAMP-specific PDE without emetogenic activity: new sulfide-like PDE7 inhibitors.

García AM, Brea J, Morales-García JA, Perez DI, González A, Alonso-Gil S, Gracia-Rubio I, Ros-Simó C, Conde S, Cadavid MI, Loza MI, Perez-Castillo A, Valverde O, Martinez A, Gil

J Med Chem. 2014 57(20):8590-607

doi: 10.1021/jm501090m.

García AM, Brea J, Morales-García JA, Perez DI, González A, Alonso-Gil S, Gracia-Rubio I, Ros-Simó C, Conde S, Cadavid MI, Loza MI, Perez-Castillo A, Valverde O, Martinez A, Gil C. Modulation of cAMP-specific PDE without emetogenic activity: new sulfide-like PDE7 inhibitors. J Med Chem. 2014 Oct 23;57(20):8590-607. doi: 10.1021/jm501090m