Marc Ten Blanco

NEW ADVANCES IN THE NEUROBIOLOGICAL MECHANISMS REGULATING FEAR EXTINCTION

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Thesis Director:
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“***Que pour examiner la vérité il est besoin, une fois dans sa vie, de mettre toutes choses en doute autant qu’il se peut.***”

René Descartes

“***Que tot està per fer i tot és possible.***”

Miquel Martí i Pol

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Quan som petits sovint somiem amb allò que creiem que ens farà feliços. Potser som uns somiatruites, potser és la tendra innocència... però potser també ens acosta a lluitar per allò que ens omple de debò. Malgrat el pas del temps i aquesta dosi de realisme que tant ens enorgulleix assolir, no hauríem de defallir mai en l'intent d'aconseguir allò que sempre hem somiat. I aquest hedonisme val per tots els aspectes de la vida, absolutament tots.

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en un momento muy duro de mi vida. Por todo ello y por muchas otras cosas que no terminaría de escribir… *muchas gracias!*

I si giro la vista enrere, veig el laboratori de **Neurophar** de la Pompeu Fabra, on vaig aprendre realment què vol dir “fer ciència”. Un grup de recerca gran, situat en front de la platja, amb uns investigadors i una infraestructura de primer nivell, amb unes festes i partits de vòlei brutalment divertits, però sobretot amb una persona que em va ensenyar a moure’m pel laboratori i l’estabulari amb seguretat. **Rocío**, en deute amb tu: mentora i bona amiga. Des que era estudiant de 2n de carrera i tu encara feies la tesi, que em vas introduir en aquest laboratori. Jo era com un pollet que anava darrere la seva mare (científica) per aprendre tot el que podia. I ara, vist en perspectiva i des del teu lloc, he de dir que ho vas fer meravellosament bé. Probablement estic acabant d’escriure aquesta tesi perquè tu em vas ensenyar, no només a agafar un ratolí, fer una immuno o quantificar espines dendrítiques, sinó també a entendre la ciència com un joc en el que ens ho podem passar molt bé, i de retruc, contribuir a millorar la societat. I més endavant... festes, congressos (avió inclòs), tornejos de vòlei, sopars, Madrid, Gavà i molts més esdeveniments que de ben segur viurem en el futur. De tot cor... *moltes gràcies!*

Pero cuando miro el presente o el pasado más cercano, veo a muchísimas personas que han pasado o todavía están en el edificio E de la UFV y que han contribuido a hacer de esta uni, un lugar en el que sentirme como si estuviera en casa. Los primeros que habitabais el laboratorio fuisteis un referente para mí, en lo profesional y en lo personal. **Noelia**, sin duda aquella persona en la que fijarme cómo se desenvolvía en el trabajo, cómo afrontabas los problemas que pudieran surgir y cómo combinabas todo esto con esa madurez y racionalidad que ya te vienen dadas por defecto. Y además proveniente de esa ciudad que tan idealizada tengo… igual tú también has tenido algo que ver. **Gonzalito** has sido mi compañero de aventuras en el lab, en el gym, en congresos, en barbacoas… y espero que lo sigas siendo aún estando en Milwaukee o donde sea que nos encontremos en el futuro. Eres brillante profesionalmente, pero todavía más como persona. Como he dicho muchas veces a otros amigos y a ti también, eres el paradigma de la bondad. Si algo he descubierto y aprendido de ti durante estos años, es que tiendes siempre a hacer el bien en los demás de forma desinteresada. Exactamente igual que tú **Marieta**, otra muy buena persona que he descubierto en Madrid y espero llevarme siempre allí donde vaya, aunque no me responda en gallego. Siempre es más fácil para mí congeniar con alguien a quien le fascina la ciencia tanto como a ti y me he contagiado de ello, estoy seguro. Pero si además compartimos fiestas de navidad de la ufv (con un final afrutado en mi casa), viernes de cervezas en Chamberí, escapadas por la sierra y

otras mil historias, hace que todo esto sea más fácil y divertido, probablemente aquellos adjetivos que mejor encajen para los momentos que estoy contigo. Y en este mismo lab también estabas tú, **Camino**. Aquella estudiante de farmacia que con tanta soltura se desenvolvía por el laboratorio de investigación, que tan buen clima ha creado, y que probablemente tenga mucho que decir de los planes y de la cohesión que hemos hecho todos juntos a lo largo de estos años. **Irene**, la post-doc y la voz de la experiencia del laboratorio a quien hacía bien en escuchar. Has sido otro de mis referentes en la investigación, con una gran resiliencia ante situaciones desfavorables, la cabeza en lo tuyo en todo momento, pero también un ojo en lo de los demás para echar una mano cuando así te lo pedíamos. Y fuera del lab descubrí a una persona con sensibilidad y empatía, con nuestros paseos durante el confinamiento por Alcobendas, los viajes a primera hora de la mañana compartiendo coche, luego al gym… y ahora seguimos y seguiremos viéndonos por videollamada. También te quiero agradecer a ti **Alejandro**, mi compi de mesa y de algún que otro curso los viernes a horas intempestivas, ese humor irónico que en muchas ocasiones me ha hecho reír un buen rato mientras el resto se preguntan si estoy bien o me pasa algo. A todos vosotros… *muchas gracias!*

Ahora la gente que está en el laboratorio ha cambiado. Sois personas que habéis empezado vuestra tesis doctoral después de mi y que seguro terminaréis demostrando a vuestro tribunal la excelencia con la que lo habéis hecho a lo largo de estos años. Y es que cuando pienso en organización, constancia y saber hacer, pienso en ti **Cris**. Esa estudiante que vino al laboratorio a ver como yo trabajaba mientas hacía la tesis, que preguntaba sin ningún reparo lo que creía que le ayudaría a entender mejor esto de la ciencia y que acabó siendo la siguiente predoc de nuestro grupo, de forma más que merecida. Durante estos años, hemos sido como un matrimonio, que se discute y se quiere a la vez. Que habla de la investigación y del mundo que hay fuera de la investigación. Que conoce a sus familias, que se van a Málaga o a Pamplona de congreso, que se llaman incluso cuando se acaban de ver en el trabajo… en breves me iré, pero sabes sin tener que decírtelo que cuentas conmigo, en lo profesional y en lo personal. También quiero agradecer a los demás miembros de este grupo de investigación/familia. Y aquí estáis **Inma**, **Rosa** y **María**. Todos juntos, cada uno con sus mejores virtudes, hacemos que la investigación avance, a la vez que nos sentimos cómodos haciéndolo. **Inma**, aportando ese punto de responsabilidad en la ciencia, tanto en la poyata como en el animalario, has sido un punto de apoyo en todos los proyectos del grupo a pesar de ir hasta arriba con la docencia. Y esto es de agradecer. **Rosa**, ojalá hubieras entrado en el grupo mucho antes. Ya te consultaba cómo poner a punto genotipajes o la multiplex… yo creo que en el fondo sabíamos que acabarías en con

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I ara sí, és clar, la meva família. Especialment els **papes**, ma **germana**, els **tiets** i **cosins**. Des de petit em dieu que soc molt familiar perquè m'agrada quan ens ajuntem tots plegats i fem dinars o sopars, aniversaris, mones de Pasqua, o passem uns dies junts a Galícia. Sempre m'ha agradat fer-ho, és veritat, però en els darrers anys des que no visc a casa encara m'ha vingut més de gust i he valorat molt més aquests moments. M'hi trobo còmode, no ens jutgem i ens estimem. Això no passa a tot arreu. Gràcies per haver estat en un dels pitjors moments, per estar a Barcelona quan pujava els caps de setmana, però sobretot... gràcies pels *tupper* que m'he endut amb menjar boníssim fet a casa i que eren l'enveja de tot el tren quan tornava a Madrid. *Moltes gràcies!*

No ho dic massa sovint, però quan escric o penso en "estimar" de de debò, sempre em venen al cap dues persones que de ben segur se sentirien molt orgulloses del seu net, l'**avi** i la **iaia**. Igual d'orgullós que em sentiria jo de poder compartir amb vosaltres aquest moment. Llàstima que ja no us tinguem amb nosaltres. Allà on sigueu, *que us acaronin els estels*.

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Quan vaig estudiar Biologia Humana vaig conèixer un grapat de persones fantàstiques que em van ajudar que tot aquest procés fos més engrescador. Han passat uns quants anyets des de llavors però segueixo valorant moltíssim l'amistat que tinc amb gent com vosaltres **Aida**, **Carla** i **Marc**. I cadascú amb el seu rol intacte! La responsabilitat i el seny que tenies i segueixes tenint, **Carla**. Aquella esfera de "res pot sortit malament" que es crea quan ets a prop. Els riures, la transigència i el bon ambient que apareix quan arribes **Marc**. Abans de trobar-nos ja em canvia la cara, somrient i pensant amb les 50.000 bromes i tonteries que ens direm, alhora que parlem del més important per a nosaltres. I tu **Aida**... des que vam començar a estudiar junts que em sorprens, sento un reforç positiu, t'admiro i em reafirmo en lo molt que t'estimo. I aquest patró es repeteix *n* vegades al llarg dels anys. Intel·ligència emocional i intel·lectual. En el teu cas no s'entendria una sense l'altra, deixaries de ser tu. Saps la satisfacció de quan publiques un article al que has dedicat molt temps i esforç, i en el que t'ho has passat molt bé mentre ho feies i et plantejaves com fer perquè fos encara millor? Doncs molt semblant és el que em passa amb tu, ets el meu *Nature*! Potser m'estic passant amb aquestes analogies tan frikis... va, prou. Al llarg d'aquests 5 anys hi ha hagut estius a Mallorca, a Galícia, setmanes teletreballant a Madrid i molt més que s'ha anat donant durant aquest temps a distància, assumint sempre que les decisions i moments rellevants han d'anar de la teva mà, és indestriable... *moltes gràcies!*

I si algú em coneix i conec des que érem adolescents i ens menjàvem el món, sou voaltres **Saray**, **Vero** i **Judy**. Quan ens ajuntem, tots 3 junts o per separat, es crea un ambient com de sopars i dinars de família, d'aquella gent que es coneix fa molts anys i

sap que tot i els problemes que puguin aparèixer, mai deixarà de ser-hi. Durant els anys de tesi, **Vero** has estat la racionalitat i bogeria alhora que m'ha fet veure punts que jo mai arribaria, mentre em feies riure i em proposaves plans alternatius que em feien i em fan sentir més viu. Estic segur que ens queden moltes vacances per tornar a passar junts. **Saray** has sido el sentido común y la responsabilidad que tan necesarios son a diario durante esta carrera de fondo en la que los dos nos encontramos inmersos. Poco hablamos de esa escapada tan bucólica a tu casita en Suiza contigo y con Muxu (quiero repetir). Y tú **Judy** has sido ese elemento imprescindible para que un engranaje funcione bien y fluya con naturalidad. Has venido a Madrid en más de una ocasión y nos hemos visto en Barcelona en muchas otras, y siempre con ganas de escucharnos, ponernos al día y volver a vernos. I això només és un 1% del que puc escriure en aquestes línies. Ara la meva tesi, però més endavant la de la Saray, el MIR de la Vero i les oposicions de la Judy. I estic segur que ho seguirem celebrant tots 3 junts i compartint els èxits com si fossin nostres... *moltes gràcies!*

Y por último también quiero agradecer a todas las personas con quien he convivido en el barrio del Plantío de Madrid, mi primer hogar fuera de casa de mis padres, y que habéis hecho a diario que este proceso fuera todavía más plácido, ameno y divertido. Especialmente a vosotros, **Nachete** y **Cata**, con todas las fiestas en casa (incluyendo piscina Cata), fuera de casa, viajecito a Málaga, el Escorial… o simplemente comidas y cenas juntos en casa. Siempre que pienso en ello, siento nostalgia. Es algo que me entristece no tener ahora, pero que valoro infinitamente haber tenido y compartido con vosotros… *muchas gracias!*

Tot i no poder estendre'm més i deixar-me moltes altres persones que en moments donats també heu jugat un paper molt important, m'ha agradat molt escriure aquest petit apartat de la meva tesi amb informació que mai podré trobar a cap article de cap revista científica, per molt factor d'impacte que tingui. I això té un valor immens que estic segur ens agradarà tornar a llegir quan siguem més grans.

MOLTES GRÀCIES A TOTS!!! MUCHAS GRACIAS A TODOS!!!

ABSTRACT

Exposure to traumatic life events might give rise to the onset of fear-related disorders, such as posttraumatic stress disorder, panic, and phobias. However, risk for such neuropsychiatric conditions varies greatly among individuals, with particular significance in genetic and epigenetic factors. For that reason, it is important to elucidate the involvement of common environmental insults on its own or in combination with other stimuli, in fear response. Animal models constitute a useful tool for the advancing in our understanding of risk factors for extinction dysregulations at the neural, genetic, and neurochemical levels. By using different behavioural and biochemical techniques, the current thesis analyses long-term effects of concomitant Δ^9- tetrahydrocannabinol (THC), the main psychoactive compound of cannabis, and stress exposure during adolescence, in the extinction of fear memories. We report that adolescent male mice simultaneously exposed to THC and stress presented fear extinction deficits in the adulthood. These fear dysregulations were paralleled with decreased neuronal activity in the main areas regulating such function and structural plasticity alterations. Once fear becomes disrupted, deciphering the biological underpinnings allows the identification of novel targets to further develop better and more effective treatment strategies. A substantial body of evidence has grown over the last years to emphasize the role of the orexin system in the modulation of fear extinction, although the underlying neurobiological mechanisms remain poorly understood. Given the neuroanatomical and functional overlapping between the orexin and the endocannabinoid system, molecular interactions in the context of impaired fear extinction induced by orexin-A have been addressed in the present thesis. We discovered a novel mechanism involving the endocannabinoid 2-arachidonoylglycerol and cannabinoid type-2 receptor (CB2R) located in the amygdala in the impairment of fear extinction induced by an overactivation of the orexin system. On the other hand, we have also evaluated the role of the endocannabinoid system in 129S1/SvImJ mice, an inbred mouse strain with remarkable fear extinction deficits. In accordance with the previous study, CB2R was markedly increased in the main brain regions that modulate fear extinction of such mouse strain, compared to C57BL/6J control mice. Pharmacological modulation of CB2R revealed an anxiolytic effect induced by CB2R blockade, whereas CB2R agonism potentiated basal-increased sensorimotor gating and fear extinction deficits, in this mouse model of aberrant fear extinction. The findings of the current thesis warn about an unknown risk factor for the correct extinction of aversive memory, consisting of simultaneous THC and stress exposure in early life stages. Moreover, CB2R is reported to play an important role in fear extinction, thus becoming a novel potential target for the treatment of fear-related disorders.

RESUMEN

La exposición a eventos traumáticos a lo largo de la vida puede dar lugar a la aparición de trastornos relacionados con el miedo, como el trastorno de estrés postraumático, pánico y fobias. Sin embargo, existe una gran variabilidad interindividual en el riesgo de sufrir dichas patologías, siendo de especial importancia los factores genéticos y epigenéticos. Por ello, es muy relevante conocer la implicación de ciertos factores ambientales nocivos en la extinción del miedo, por sí mismos o en combinación con otros. Los modelos animales son una herramienta útil para avanzar en el conocimiento de los factores de riesgo para la desregulación en la extinción del miedo a nivel neuronal, genético y neuroquímico. Mediante test de comportamiento y técnicas bioquímicas, esta tesis evalúa los efectos a largo plazo de la exposición a Δ^9- tetrahidrocannabinol (THC), principal componente psicoactivo del cannabis, y estrés durante la adolescencia, en la extinción de memorias aversivas. Hemos observado que los ratones macho expuestos simultáneamente a THC y estrés en la adolescencia presentaron déficits en la extinción del miedo cuando eran adultos. Estas alteraciones se asociaron con una hipoactividad en las principales áreas que regulan dicha función y con alteraciones en la plasticidad sináptica. Una vez se desregula el miedo, conocer los mecanismos subyacentes permite la identificación de nuevas dianas terapéuticas para mejorar el tratamiento actual. En los últimos años, el rol del sistema de orexinas en la extinción del miedo ha ganado importancia, aunque los mecanismos neurobiológicos todavía se desconocen. Dadas las coincidencias neuroanatómicas y funcionales de los sistemas de orexina y endocannabinoide, en esta tesis se han abordado las interacciones moleculares subyacentes a las alteraciones en la extinción del miedo inducidas por la orexina-A. Hemos descubierto un nuevo mecanismo en la alteración de la extinción del miedo debido a la sobreactivación del sistema de orexinas, que incluye el endocannabinoide 2-araquidonoilglicerol y el receptor cannabinoide tipo 2 (CB2R) en la amígdala. Por otro lado, hemos evaluado el papel del sistema endocannabinoide en ratones 129S1/SvImJ, una cepa con alteraciones en la extinción del miedo. De acuerdo con el estudio anterior, estos animales presentaron un aumento de CB2R en las principales regiones que modulan la extinción del miedo, en comparación con la cepa control. El bloqueo de dicho receptor indujo un efecto ansiolítico, mientas que su agonismo potenció el incremento basal del filtro sensoriomotor y los déficits en la extinción del miedo. Los hallazgos de esta tesis alertan sobre un nuevo factor de riesgo para la correcta extinción del miedo, la exposición simultánea a THC y estrés en la adolescencia. Además, se presenta CB2R como un elemento importante para la extinción del miedo, postulándose así como una posible diana terapéutica para el tratamiento de trastornos relacionados con el miedo.

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
ACTH	adrenocorticotropic hormone
AD	Alzheimer disease
AEA	anandamide
BDNF	brain-derived neurotrophic factor
BL6	C57BL/6J
BLA	basolateral amygdala
BNST	bed nucleus of stria terminalis
CB1R	cannabinoid type-1 receptor
CB2R	cannabinoid type-2 receptor
CBD	cannabidiol
CNS	central nervous system
CRF	corticotropin-releasing factor
CS	conditioned stimulus
CSF	cerebrospinal fluid
DAGL	diacylglycerol lipase
DORA	dual orexin receptor antagonist
DSM	Diagnostic and Statistical Manual of Mental Disorders
EMA	European Medicines Agency
FAAH	fatty acid amide hydrolase
FDA	Food and Drug Administration
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
HPA	hypothalamic-pituitary-adrenal

ICV intracerebroventricular

IL infralimbic

KO knockout

LC locus coeruleus

LHA lateral hypothalamic area

MAGL monoacylglycerol lipase

mPFC medial prefrontal cortex

NAc nucleus accumbens

NAPE N-arachidonoyl-phosphatidylethanolamine

OX1R orexin receptor-1

OX2R orexin receptor-2

OXA orexin-A

OXB orexin-B

PAG periaqueductal gray

PD Parkinson disease

PL prelimbic

PLC phospholipase C

PPI prepulse inhibition

PTSD posttraumatic stress disorder

PVN paraventricular nucleus of the hypothalamus

S1 129S1/SvImJ

SORA single orexin receptor antagonist

THC Δ^9- tetrahydrocannabinol

TMN tuberomammillary nucleus

US unconditioned stimulus

VTA ventral tegmental area

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INTRODUCTION

1. CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM

Humans have consumed cannabis derivates over thousands of years due to their medicinal and recreational use. From them on, more than 100 cannabinoids have been isolated from the *Cannabis sativa* plant, with particular attention to the main psychoactive compound Δ^9-tetrahydrocannabinol (THC), discovered in the 1960s by Mechoulam and Gaoni (Mechoulam and Gaoni, 1965). This seminal discovery opened the door to the exploration of a novel neuromodulatory system, the endocannabinoid system. Over the next decades, Devane and co-workers identified the first cannabinoid receptor in rat and human brains, by using a synthetic radiolabelled THC analogue (Devane et al., 1988). This receptor was known to be the major site of action of THC and other cannabinoids. Subsequently, a similar cannabinoid receptor highly expressed in the immune system was discovered by homology cloning (Matsuda et al., 1990; Munro et al., 1993). A crucial breakthrough was the identification of the two main endogenous ligands of both cannabinoid receptors, which were referred to as endocannabinoids (Devane et al., 1992; Mechoulam et al., 1995). Then, the close association between cannabis and humans made sense, since it was known that human brain is able to produce and process cannabinoids.

Cumulative knowledge has established the endocannabinoid system as a widespread neuromodulatory network, involved in a plethora of physiological and cognitive processes. This system consists of the endocannabinoids, their receptors, and the enzymatic machinery responsible for synthesising and degrading these endocannabinoids. Moreover, diverse mediators biochemically related to the endocannabinoid system components have been discovered at a later time, thus expanding the primarily known endocannabinoid system, which is named endocannabinoidome (di Marzo and Wang, 2015). This complex network overlaps with other pathways and includes different metabolic processes. From a functional perspective, the endocannabinoid system acts retrogradely from the postsynaptic to the presynaptic neuron and is broadly expressed throughout the entire central nervous system (CNS) (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Busquets-Garcia et al., 2018). For that reason, it has been demonstrated a key role in several biological functions, including brain development, pain, stress coping, motivation, and energy expenditure, among many others (Cristino et al., 2020).

1.1. Overview of the endocannabinoid system

As previously explained, the endocannabinoid system is composed of receptors, the two main endocannabinoids, and the enzymes involved in the synthesis and degradation of each endocannabinoid.

1.1.1. Cannabinoid receptors

Endogenous and exogenous cannabinoids activate at least two different receptors, the cannabinoid type-1 and type-2 receptors (CB1R and CB2R, respectively). Both belong to the G protein-coupled receptor (GPCR) family, specifically coupled to Gi/o proteins. However, increasing evidence indicates that other receptors also bind cannabinoid ligands, such as peroxisome proliferator-activated receptor-α (PPARα) (O'Sullivan, 2007), orphan GPCR 55 (GPR55) (Lauckner et al., 2008; Godlewski et al., 2009), orphan GPCR 119 (GPR119) (Godlewski et al., 2009) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Di Marzo and De Petrocellis, 2010), among others. Interestingly, these receptors often have opposite roles to those of CB1R and CB2R (Kawahara et al., 2011; Benito et al., 2012; Hansen et al., 2012).

CB1R is a seven-transmembrane domain receptor, which is mainly expressed in the brain. Indeed, it is claimed to be the most abundant GPCR in the mammalian brain (Cristino et al., 2020). Neuroanatomical distribution of CB1R has been profoundly characterized in both rodents (Herkenham et al., 1991; Tsou et al., 1998) and humans (Westlake et al., 1994; Burns et al., 2007), with a high density in almost all brain regions, especially in the cerebellum, basal ganglia, and hippocampus (Figure 1). However, CB1R is not a nervous system-restricted receptor, as it is also expressed in peripheral tissues including heart, lung, adrenal glands, retina, liver, gonads, adipocytes and immune and vascular systems (Pertwee et al., 2010). Advanced microscopy technology, such as electron microscopy, has better identified CB1R at the cellular level. In this sense, CB1R is predominantly expressed presynaptically in both excitatory and inhibitory neurons, where it inhibits voltage-gated Ca^{2+} channels and vesicular release of γ-aminobutyric acid (GABA) or glutamate (Caulfield and Brown, 1992; Mackie and Hille, 1992). Hence, CB1R acts as a retrograde modulator of different types of neurotransmitters in the synaptic process (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Additionally, some evidence indicates that CB1R is also located in astrocytes where it regulates synaptic plasticity (Sánchez et al., 2001; Robin et al., 2018), and even in the external membrane of mitochondria by inhibiting electron transport and the respiratory chain (Bénard et al., 2012; Hebert-Chatelain et al., 2016). For that reason,

CB1R has emerged as a potential therapeutic target for many diseases, given its involvement in a wide variety of physiological functions.

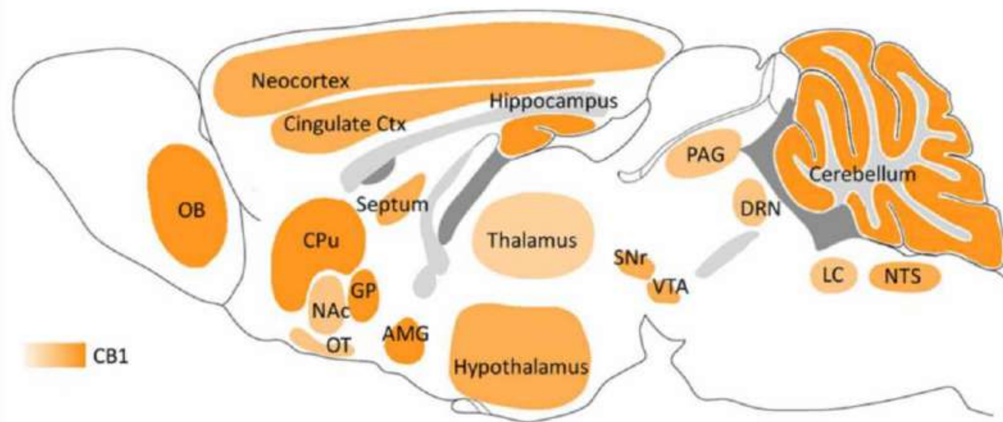

Figure 1. Schematic representation of the main areas expressing CB1R. Colour intensity reflects CB1R expression levels. AMG, amygdala; CPu, caudate putamen; Ctx, cortex; DRN, dorsal raphe nucleus; GP, globus pallidus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area (Adapted from Flores *et al*, 2013).

On the other side, CB2R is also a seven-transmembrane domain GPCR, which central function is the modulation of the immune system (Cristino et al., 2020). Cumulative knowledge indicates that CB2R is primarily found in peripheral tissues, mainly in immune cells (Munro et al., 1993; Galiègue et al., 1995; Brown et al., 2002; Liu et al., 2009; Simard et al., 2022). Unlike CB1R, CB2R expression in the CNS is low under physiological conditions, and it can be increased in concrete pathological states, including schizophrenia, depression, addiction, and Parkinson disease (PD), among others, thus becoming a potential therapeutic target for several CNS diseases (Kibret et al., 2022). Nevertheless, its cell-type location in the CNS presents unresolved controversies due to the questioned selectivity of CB2R antibodies (Atwood and MacKie, 2010; Cabañero et al., 2021). A broad consensus is emerging on the important role of CB2R in microglial cells (Komorowska-Müller and Schmöle, 2020; Reusch et al., 2022; Ruiz de Martín Esteban et al., 2022) and, to a lesser extent, in astrocytes (Stella, 2010; Jia et al., 2020). Importantly, its expression in these glial cells is significantly increased in chronic neuroinflammation-associated diseases (Cabral and Griffin-Thomas, 2009), such as Alzheimer disease (AD) (Benito et al., 2003). Contrary to the wide agreement on microglial expression of CB2R, reasonable concerns remain on the CB2R location in neurons. While early studies showed that CB2R was absent in neuronal cells (Munro et al., 1993), further research indicates that it is present in neurons of several brain areas,

including hippocampus, amygdala, thalamus, striatum, cerebral cortex, cerebellum, and spinal and olfactory nuclei (van Sickle et al., 2005; Onaivi et al., 2006; Jordan and Xi, 2019). Moreover, the mechanism by which CB2R regulates neuronal function remains unknown, in contrast to the well-characterized CB1R. On balance, there is clear consistency among research studies indicating an inducible character for CB2R mainly in pathological conditions, whereas its function and cell-type location need to be further explored and constitute one of the specific objectives of the present thesis (Articles 2 and 3).

Cannabinoid receptors are involved in a wide range of functions by activating different signal transduction pathways (Ye et al., 2019). In particular, both CB1R and CB2R act through the activation of Gi/o proteins (Howlett and Abood, 2017), although CB1R might also bind Gs and Gq proteins in specific conditions (Glass and Felder, 1997; Lauckner et al., 2005). While CB1R signalling has been extensively studied, only a few studies have addressed CB2R to date. Then, CB1R stimulation regulates the activity of diverse ion channels, including K^+ and Ca^{2+} channels, in order to repolarize the plasmatic membrane and inhibit the release of neurotransmitters (Deadwyler et al., 1995; Vásquez et al., 2003). Also, the activation of CB1R mediates the inhibition of adenyl cyclase and, in turn, decreases cAMP and reduces the activity of protein kinase A (Howlett, 2005). This activation might also initiate other kinase signalling cascades, such as the phosphoinositide 3-kinase pathway, glycogen synthase kinase 3 and protein kinase C (Bouaboula et al., 1995; Gómez Del Pulgar et al., 2000; Ozaita et al., 2007), thus modifying gene expression (Figure 2). Given the complexity of endocannabinoid signalling, allosteric modulators of CB1R and CB2R have emerged as promising tools. Thereby, several downstream pathways might be modulated, while preserving the site- and time-selectivity of endocannabinoids at these receptors and avoiding any interference with other cannabinoid mediators (Bosier et al., 2010).

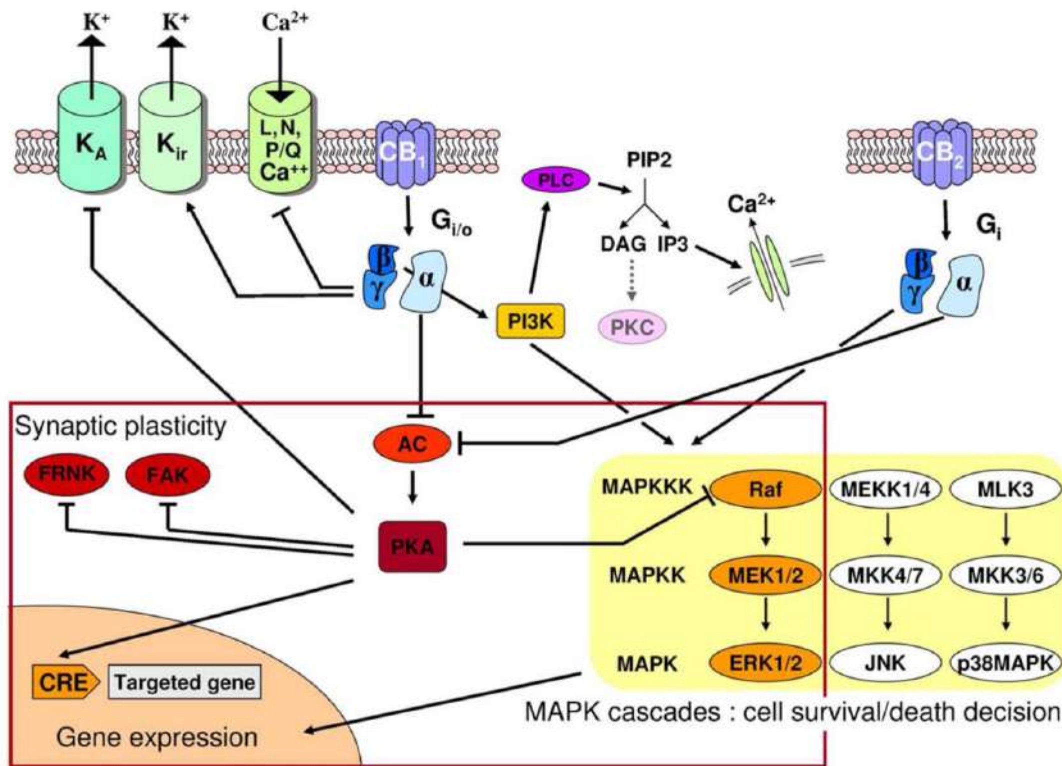

Figure 2. Regulation of cell excitability and neurotransmitter release through CB1R. (Adapted from Bosier *et al*, 2010).

1.1.2. Endocannabinoids

Endogenous cannabinoids, also named endocannabinoids, are signalling lipids that activate cannabinoid receptors. The two main endocannabinoids are anandamide (the ethanolamide of arachidonic acid) and 2-arachidonoylglycerol, often abbreviated as AEA and 2-AG, respectively (Figure 3). Nevertheless, other endocannabinoid-related molecules, such as 2-arachidonyl glyceryl ether (Hanus et al., 2001) and N-arachidonoyl dopamine (Grabiec and Dehghani, 2017), also engage cannabinoid receptors.

AEA was the first known endocannabinoid (Figure 3), isolated from porcine brain. Its name was coined from the Sanskrit word *ananda* meaning “bliss”, and from the chemical nature of this compound (Devane et al., 1992). This endocannabinoid acts as a partial agonist at both cannabinoid receptors, although it presents higher affinity for CB1R rather than for CB2R (Reggio, 2010). A few years later, 2-AG was discovered as the second endogenous cannabinoid ligand (Mechoulam et al., 1995; Sugiura et al., 1995) (Figure 3). This endocannabinoid behaves as a full agonist for both CB1R and CB2R with higher potency than AEA, and presents the characteristic effects associated with cannabinoid agonism (Reggio, 2010). Interestingly, 2-AG has been found in the brain at concentrations 170 times greater than AEA (Stella et al., 1997). Both endocannabinoids

present a similar distribution pattern in the CNS, with high density in the brainstem, striatum and hippocampus, and lower in the diencephalon, cortex and cerebellum (Bisogno et al., 1999). As previously mentioned, other putative endocannabinoids with lower affinity for both cannabinoid receptors have also been characterized. However, their functional relevance remains to be deciphered (Cristino et al., 2020).

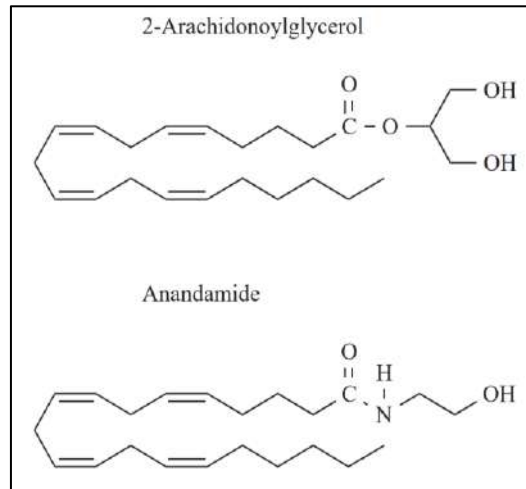

Figure 3. Structure of the endocannabinoids 2-arachidonoylglycerol and anandamide. (Adapted from Fisar, 2009)

A distinctive feature of the endocannabinoid system is its mechanism of action. Endocannabinoids are synthesized and released from the postsynaptic terminals in an activity-dependent manner, and travel through the synaptic cleft to the presynaptic axon, thus binding CB1R (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). As a neuromodulatory system, this retrograde signalling produces a transient decrease of the release of other neurotransmitters, in order to avoid an uncontrolled overactivation of any neurotransmission system (Caulfield and Brown, 1992; Mackie and Hille, 1992; di Marzo et al., 2005). As detailed below, such modulatory function of both AEA and 2-AG needs to be accurately regulated through their correspondent pathways of synthesis and degradation.

1.1.3. Enzymes involved in the synthesis and degradation of endocannabinoids

In contrast to classic neurotransmitters, endocannabinoids are synthesised “on-demand” since their precursors exist as membrane lipids and are liberated by the activation of the correspondent enzymes (di Marzo et al., 1999). Such activation occurs in response to concrete signals, such as increased levels of intracellular Ca^{2+} (di Marzo et al., 1994; Placzek et al., 2008) or the activation of a GPCR (Vellani et al., 2008; Wu et al., 2020). Hence, both AEA and 2-AG are accurately released in a precise time and space.

There are several pathways for the synthesis of endocannabinoids. The importance of each one depends on specific factors, such as the tissue, the development state and the presence or absence of any pathological condition (Lu and Mackie, 2021). However, a more prominent pathway has been widely described for each endocannabinoid. The synthesis of AEA is mainly accomplished through the hydrolysis of its phospholipid precursor N-arachidonoyl-phosphatidylethanolamine (NAPE) by a specific phospholipase D, although additional pathways are well described (di Marzo et al., 1994; Biringer, 2021). On the other hand, the canonical pathway of 2-AG synthesis involves the hydrolysis of its precursor diacylglycerol, by the enzyme diacylglycerol lipase (DAGL) (Mechoulam et al., 1995; di Marzo et al., 2005). This enzyme presents two different isoforms, DAGL-α and DAGL-β (Bisogno et al., 2003). Both are highly expressed in the entire CNS, with slight differences in their cell type location. Whereas DAGL-α takes an important role in the synaptic production of 2-AG, DAGL-β is primarily involved in the synthesis and release of 2-AG from microglial cells (Viader et al., 2016).

Once endocannabinoids have activated their molecular targets, both AEA and 2-AG are removed from the synaptic cleft. Although little is known about endocannabinoid trafficking across the cell membrane, recent evidence points carrier-mediated facilitated diffusion, such as Na^+- or ATP-requiring transporters, as the most likely mechanism for endocannabinoids transport (Kaczocha and Haj-Dahmane, 2022). Subsequent degradation is carried out by specific intracellular enzymes, which hydrolyse the arachidonic acid from either the ethanolamine and the glycerol of AEA and 2-AG, respectively. AEA is hydrolysed by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996, 2001), an enzyme mainly found in the soma and dendrites of postsynaptic neurons (Egertová et al., 2003). Analogously, 2-AG hydrolysis is exerted by monoacylglycerol lipase (MAGL) or α/β-hydrolase domain containing 6 (ABHD6) (Dinh et al., 2002; Marrs et al., 2010). MAGL is primarily expressed in presynaptic terminals, while ABHD6 is mostly found in dendrites (Gulyas et al., 2004; Marrs et al., 2010; Ludányi et al., 2011).

1.1.4. Physiological functions

Since its discovery in the 1990s, the endocannabinoid system has emerged as an essential modulator of several physiological, behavioural, immunological, and metabolic functions, given its extensive distribution across almost all tissues, especially in the CNS. As explained before, cannabinoid receptors are expressed in a region-specific manner. However, considering both CB1R and CB2R distribution, the endocannabinoid system is involved to a greater or lesser extent in the modulation of a wide variety of physiological and pathological conditions (Zou and Kumar, 2018).

In the central and peripheral nervous system, the endocannabinoid system acts as an essential player in the regulation of the synaptic homeostasis. Hence, it modulates synapse creation and remodelling (Kano et al., 2009), and specific processes that modulate neuronal development, such as neuronal differentiation, proliferation, migration, and survival (Rueda et al., 2002; Harkany et al., 2008; Galve-Roperh et al., 2013). In this vein, the endocannabinoid system regulates nociception under different types of acute and chronic pain (Finn et al., 2021). Moreover, CB1R in the cerebellum and basal ganglia has been linked with motor coordination and cerebellar learning performance (el Manira and Kyriakatos, 2010). This receptor in the hippocampus has also been investigated due to cannabis effects on memory and learning (Puighermanal et al., 2009; Kloft et al., 2020). It is also remarkable the role played by this neuromodulatory system to ensure an appropriate reaction to stressful situations, thus controlling anxiety and fear responses (Maldonado et al., 2020). Moreover, the endocannabinoid system is widely known to regulate rewarding properties of diverse substances and events, given its broad expression in the limbic system and related brain areas (Mechoulam and Parker, 2013). For that reason, it has become an essential component to modulate addictive disorders (Maldonado et al., 2006; Spanagel, 2020).

The endocannabinoid system is also relevant in peripheral tissues. It regulates the mobility of the gastrointestinal tract, where it also modulates the secretion of gastric fluids, neurotransmitters and hormones, as well as the permeability of the intestinal epithelium and the gut microbiota (Mehrpouya-Bahrami et al., 2017; Wang et al., 2020; Izzo and Sharkey, 2010). Both CB1R and CB2R have a low expression in hepatic and cardiovascular tissues. However, CB1R is upregulated in both tissues under pathological conditions, thus becoming an interesting pharmacological target (Miller and Devi, 2011; Montecucco and di Marzo, 2012). CB2R modulates the activity of the immune system, in which cells is highly expressed (Turcotte et al., 2016; Simard et al., 2022). In addition to the aforementioned tissues, the endocannabinoid system has also been reported to have an important role in the reproductive system, skeletal muscle, adipose tissue, eye, bone, and skin, among others (Maccarrone et al., 2015).

1.2. Psychoactive cannabinoids: focus on THC

Aside from previously described endocannabinoids, several exogenous compounds can also interact with cannabinoid receptors. Such structurally diverse class of natural constituents in the *Cannabis sativa* plant are named cannabinoids, also known as phytocannabinoids due to their plant origin. Indeed, more than 500 compounds have been isolated from this plant, of which 125 are classified as cannabinoids. The remaining

compounds include terpenes, phenols, flavonoids and alkaloids, among others (Radwan et al., 2021).

THC is the main psychotropic cannabinoid in the plant (Figure 4), which activates CB1R, thus inducing the well-known cannabinoid tetrad effects: hypothermia, hypolocomotion, catalepsy and analgesia (Prescott et al., 1992; Meng et al., 1998; Smirnov and Kiyatkin, 2008; Pagano et al., 2022). This cannabinoid is considered a partial agonist of both cannabinoid receptors and exerts its psychoactive effects via CB1R agonism (Schurman et al., 2020). In contrast, the non-psychotropic cannabinoid cannabidiol (CBD) has recently gained attention (Figure 4), since it presents several beneficial attributes such as neuroprotective, anticonvulsant, anti-inflammatory, antispasmodic, and antiemetic properties, avoiding side effects (Hampson et al., 1998; Baker et al., 2000; Malfait et al., 2000; Rock et al., 2012; van den Elsen et al., 2014). Although its mechanism of action is still a controversial issue, a growing number of studies points that CBD has relatively little affinity for the orthostatic sites of both cannabinoid receptors, thus exhibiting a negative allosteric binding activity on CB1R (Laprairie et al., 2015; Tham et al., 2019; Peng et al., 2022). A similar allosteric site for CBD has been found in CB2R, which allows this compound to promote anti-inflammatory effects (Martínez-Pinilla et al., 2017; Peng et al., 2022). However, CBD has many properties that are independent of cannabinoid receptors, whose mechanisms remain unknown. Other cannabinoids, such as tetrahydrocannabivarin, cannabinol, cannabigerol, and cannabichromene are also present in the *Cannavis sativa* plant, albeit in lesser amounts in comparison with THC and CBD.

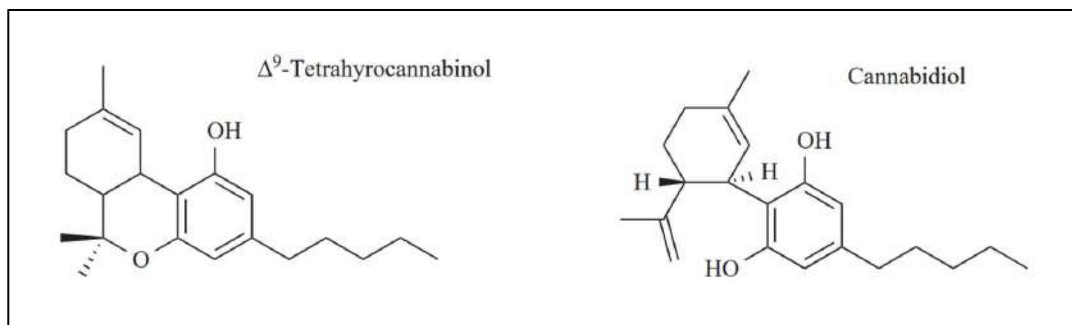

Figure 4. Structure of the phytocannabinoids Δ^{9}-tetrahydrocannabinol and cannabidiol. (Modified from Fisar, 2009)

Given the widely studied therapeutic properties of the two major cannabinoids, CBD has been already approved by several regulatory agencies for the treatment of convulsive seizures (Epidiolex), as well as the combination of CBD and THC in a 1:1 ratio for multiple

sclerosis-related spasticity, and pain associated with the same disease and cancer (Sativex). Also, nabilone and dronabinol, two synthetic cannabinoid drugs approved in diverse countries, are already used in clinical practice due to their analgesic properties for patients with chronic pain, and for reducing nauseas and vomiting in cancer patients (Pagano et al., 2022). Legalization of cannabis and derivates for medical or recreational use is a current hot topic with controversial opinions among the scientific community and society as a whole. However, it was in 1985 when dronabinol was first approved in the USA for the treatment of anorexia derived from acquired immunodeficiency syndrome (AIDS) due to human immunodeficiency virus (HIV), as well as chemotherapy-induced nausea and vomiting in patients with who had failed to respond to conventional treatments (O'Donnell et al., 2022).

Despite the increasing number of approved drugs and the growing body of evidence reporting novel therapeutic properties for THC, this cannabinoid entails psychotropic effects that impair physiological functions, to a greater or lesser extent (Volkow et al., 2016; National Academies of Sciences, 2017; Sagar and Gruber, 2018). In addition, *Cannabis sativa* plant has been largely manipulated to obtain a higher potency by increasing the THC to CBD ratio (Lafaye et al., 2017). For that reason, recreational cannabis use is related to cognitive decline and neural changes, among many other consequences, especially when consumption begins in early life stages.

1.2.1. Cannabinoids use, abuse, and dependence

Cannabis is the most consumed illicit drug worldwide, and the third drug of abuse most commonly used after alcohol and tobacco (first and second, respectively). Data from the European Drug Report of 2022 show that 66% of the total drug confiscations in Europe in 2020 corresponds to different types of cannabis preparations, far ahead of cocaine, the second most seized drug (13%) (Figure 5). As a consequence, cannabis was the substance most frequently reported by hospitals involved in the European Drug Emergency Network in 2020. About a quarter of acute drug toxicity presentations involved cannabis, frequently in the presence of other substances. Moreover, its consumption is also related to economic and gender differences. High-income countries present the highest prevalence of cannabis use, although an increasing use is being reported in low- and middle-income countries. On the other hand, a clear disparity exists between genders, since 84% of users entering a chronic cannabis consumption in 2021 were men. Another interesting fact on cannabis use in Europe was carried by the recent Covid-19 pandemic. Health measures and restrictions adopted during this period have impacted cannabis use patterns, with an increase on herbal cannabis consumption

among frequent users and a clear decrease between infrequent users (European Monitoring Centre for Drugs and Drug Addiction, 2022).

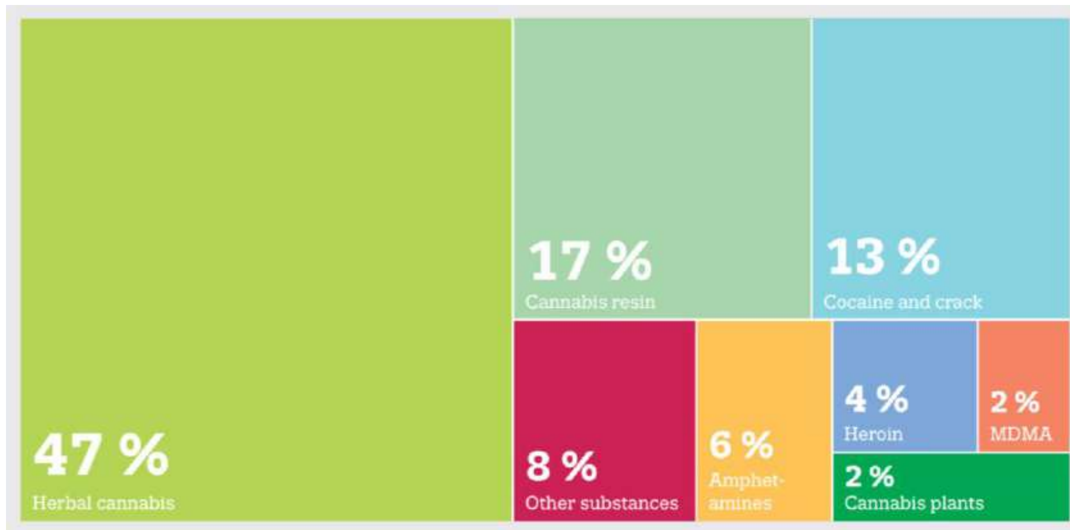

Figure 5. Number of reported drug confiscations in Europe in 2020. Cannabis-derived preparations includes herbal cannabis, cannabis resin, and cannabis plants (Adapted from European Monitoring Centre for Drugs and Drug Addiction, 2022).

There are different types of cannabis preparations, all of them contain substantial amounts of THC. Interestingly, analysis performed in 2020 revealed that the most resinous parts of the cannabis plant (traditionally named as *hashish*) presented an average THC content of 21%, almost twice that of herbal cannabis (also known as *marijuana*), at 11% (European Monitoring Centre for Drugs and Drug Addiction, 2022). These preparations are usually mixed with tobacco and the most common rout of administration is smoke inhalation. Once cannabis is consumed, individuals experience rewarding effects characterized by euphoria, lethargy, and overstretch of ordinary situations (Johnson, 1990). These effects may also be accompanied by physical signs, including tachycardia, increased appetite, and bronchodilatation, among others (Hall and Solowij, 1998; Karila et al., 2014). However, negative effects are hiding beneath this pleasant sensation. In this respect, acute cannabis use may also induce anxiety, dysphoria, disorientation, psychomotor agitation, deficits in prospective memory and executive functions, hallucinations, and paranoid ideas (Johns, 2001; Martin-Santos et al., 2012; Montgomery et al., 2012). In high doses, cannabis consumption may potentiate schizophrenia-like symptoms, leading to an acute functional psychosis (Moore et al., 2007).

About 10% of individuals that reported cannabis use in 2020 were daily or near-daily users (United Nations Office on Drugs and Crime (UNODC), 2022). Thus, chronic

cannabis consumption entails long-term consequences, such as the impairment of verbal learning and memory, working memory, and attention. Moreover, associations between stronger cognitive alterations and a younger age of onset have been regularly reported (Broyd et al., 2016). Chronic cannabis intake has also been related to other cognitive disturbances like depersonalization and amotivational syndromes, which entail an alteration in subjective experience of reality and reduced motivation and capacity for daily activities, respectively (Moran, 1986; Hürlimann et al., 2012; Volkow et al., 2016). Furthermore, different somatic disorders may appear after recurrent cannabis use. For instance, hyperemesis, asthma exacerbation, airflow obstructions, cardiac arrythmias and nasopharyngeal carcinoma, among many others (Feng et al., 2009; Galli et al., 2011; Tessmer et al., 2012; Tashkin and Roth, 2019; Richards et al., 2020). On the other hand, cannabis consumption also entails social consequences, since it has been widely related with low academic achievement, unemployment and delinquency (de Looze et al., 2015; Hernández-Serrano et al., 2018; Barry et al., 2022).

Despite the negative effects for human health, acute rewarding properties from cannabis can induce a repeated and uncontrolled consumption. This behaviour has been recently categorized as a mental illness in the last update of the main psychiatry manual, the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), referred to as cannabis use disorder (American Psychiatric Association, 2013). Such disturbance affects about 10% of cannabis users worldwide and as many as one-third of those who use it daily (United Nations Office on Drugs and Crime (UNODC), 2022). Aside from cannabis use disorder, the DSM-5 also includes cannabis intoxication and withdrawal within the framework of cannabis-related disorders, thus resembling the same features as any other drug addiction (Connor et al., 2021).

1.2.2. Cannabis exposure in early life stages

Nervous system formation and maturation comprises a vulnerable life stage, in which environmental perturbations like cannabis consumption might have a dramatic cost for individual's health. Adolescence is included in this critical period, since efficient neuronal pathways by constant neuroplastic shaping, synaptic reorganization and neurochemical changes are created (Sturman and Moghaddam, 2011). Over this period, many players are involved to achieve a correct maturation of the CNS. In particular, the endocannabinoid system has a key role through the regulation of several processes, such as the maturation of the corticolimbic circuit neurons by achieving a balance between inhibitory and excitatory neurotransmission in the prefrontal cortex (Meyer et al., 2018). These maturational events require an accurate signalling, that is reflected in

variations in the temporal expression of components of the endocannabinoid system during adolescence. Studies in rodents showed that CB1R expression peaks with the onset of adolescence with a subsequent decline approaching adulthood (de Fonseca et al., 1993; Heng et al., 2011). Interestingly, 2-AG levels remain high at the beginning and the end of adolescence with attenuated expression in mild-adolescence, whereas AEA present constant fluctuations during this period (Ellgren et al., 2008; Lee et al., 2013; Rubino et al., 2015a) (Figure 6). For that reason, direct perturbations into the endocannabinoid system, such as cannabis intake, might induce detrimental effects in the adulthood.

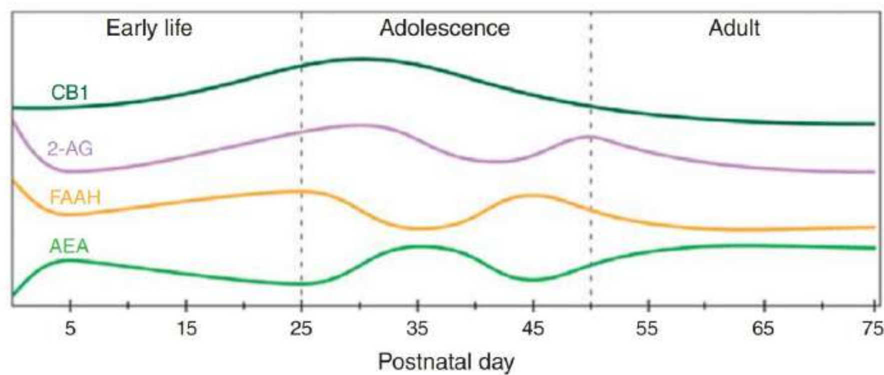

Figure 6. Developmental trajectories of the components of the endocannabinoid system. (Adapted from Meyer *et al*, 2018).

As previously mentioned, recreational use of cannabis is widespread worldwide. Specifically, cannabis consumption in 2021 among the European population aged 15-34 is estimated at 15.5%. However, this figure is even higher among adolescents aged 15-24 that is at 19.1% (European Monitoring Centre for Drugs and Drug Addiction, 2022). Preclinical and clinical studies indicate that cannabinoid exposure during this critical window of development produces short- and long-lasting neurobiological changes that affect the function and behaviour of the brain (Renard et al., 2014). In this vein, cognitive effects have been widely studied in both humans and animal models (Scheyer et al., 2022). Rats chronically exposed to THC and other CB1R agonists during adolescence presented short- and long-term memory impairment, usually evaluated by using the object recognition task, and spatial working memory deficits measured with the radial maze test (O'Shea et al., 2006; Abush and Akirav, 2012; Renard et al., 2013). The neurobiological mechanisms underlying these cognitive disruptions have been associated with diverse alterations, including changes in hippocampal morphology, as well as reduced protein expression in the same region and the medial prefrontal cortex (mPFC), mainly synaptic proteins (e.g., synaptophysin and PSD95) (Rubino et al.,

2009a, 2009b; Llorente-Berzal et al., 2013). Clinical studies recapitulate these cognitive impairments by adding deficits in cognitive inhibition, attention, impulsivity, and overall intelligence quotient (Fontes et al., 2011; Meier et al., 2012). Anxiety is one of the most common disorders developed by chronic cannabis use, with a higher prevalence in females (Hayatbakhsh et al., 2007; Kedzior and Laeber, 2014). However, only a few studies have assessed the relationship between cannabis use and long-term anxiety disorders in humans. Moreover, animal studies evaluating anxiety-like behaviour have reported inconsistent results, due to differences in the specific period in which cannabinoids are administered and the test used to assess this behaviour (Biscaia et al., 2003; Higuera-Matas et al., 2009; Saravia et al., 2019). Also, depressive and mood disorders have been strongly associated with cannabis consumption. Such disorders present a prevalence of 25% among cannabis users. However, this risk can increase by five-fold depending on the age of cannabis use onset and the gender (Grant and Pickering, 1998; Green and Ritter, 2000; Chabrol et al., 2008). Accordingly, preclinical studies with rodents treated with cannabinoids during the adolescent period presented a clear depressive-like phenotype in the adulthood, by using the sucrose preference and the forced-swim tests (Rubino et al., 2008a; Bambico et al., 2010; Zamberletti et al., 2014). Furthermore, Rubino and co-workers identified a decreased expression of CB1R in the amygdala, ventral tegmental area (VTA) and nucleus accumbens (NAc), thus associating this biochemical alteration with the depressive phenotype observed (Rubino et al., 2008a).

Not only cognitive impairments are derived from early cannabis consumption, but also long-term psychotic effects have been reported as a consequence of adolescent cannabis use (Degenhardt and Hall, 2002; Hasan et al., 2020). Indeed, in 1987 a study performed with young healthy Swedish subjects demonstrated a six-fold increased risk of developing schizophrenia later in life as a result of heavy cannabis use at age 18 (Andréasson et al., 1987). Several studies with humans have further confirmed this concerning association, thus pointing the age of onset of cannabis intake and the genetic variations as key parameters for the appearance of psychotic disorders (Arseneault et al., 2002; Stefanis et al., 2004; Hiemstra et al., 2018). In animal studies, disruptions in the prepulse inhibition (PPI) of startle reflex, which evaluates the sensorimotor gating, is widely accepted as an endophenotype of psychotic disorders (Braff et al., 2001) (Figure 7). In accordance with clinical findings, adolescent rodents treated with cannabinoids presented a decreased PPI response, thus revealing a psychotic phenotype (Schneider and Koch, 2003; Wegener and Koch, 2009; Abela et al., 2019).

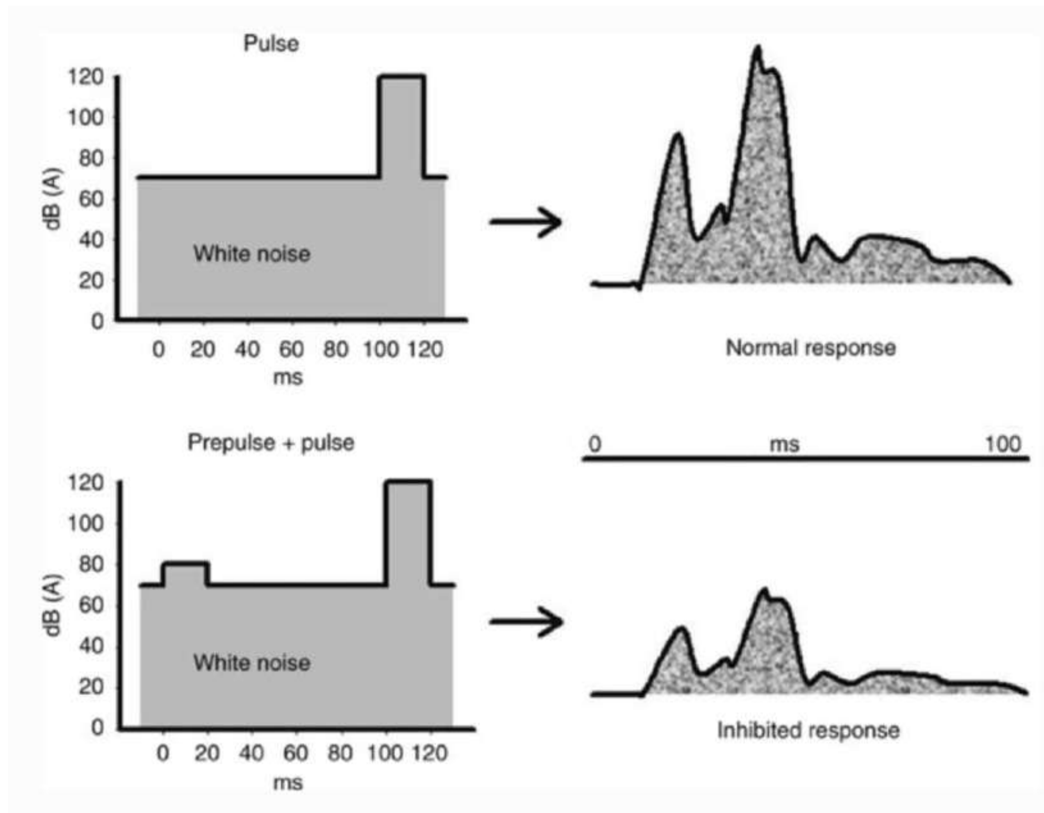

Figure 7. Diagrammatic representation of prepulse inhibition of startle reflex. (Adapted from Geyer, 2010).

In conclusion, cannabis use at early life stages, predominantly in the adolescence, increases the risk of long-term cognitive and/or psychotic effects. However, most alarming consequences arise from the synergistic effect of multiple factors, such as environmental events (e.g., stress, drug abuse) and gene polymorphisms that enhance risk (e.g., catechol-O-methyltransferase (COMT) gene) (Rubino and Parolaro, 2016). This well-known “two-hit” hypothesis has been widely accepted in the case of schizophrenia (Maynard et al., 2001; Dalton et al., 2012; Davis et al., 2016), although it remains unexplored in other phenotypes. For that reason, one of the main objectives of the present thesis is to evaluate the cumulative effect of THC consumption and stress exposure during the adolescence in the extinction of fear memories in the adulthood (Article 1).

1.2.3. Synthetic cannabinoids

Recreational use of cannabis has recurrently faced legal barriers in most countries. An alternative solution to the classic cannabis drugs is the consumption of synthetic cannabinoids, given the easy accessibility and limited availability of sensitive analytical methods for screening this group of compounds (Ford et al., 2017). Originally produced

for a research purpose, synthetic cannabinoids are currently manufactured by clandestine laboratories and sold in striking coloured packages containing a mixture of a dried base plant without psychotropic effects, such as Mentha or Melissa, and a synthetic cannabinoid usually added by soaking or spraying (Duccio et al., 2018; Alves et al., 2020). These products were initially commercialized as “Spice” in European countries and “K2” in the United States, through internet retailers and “headshops” (Seely et al., 2011). From the early 2000s, they have rapidly gained attention mainly in Western countries. Nowadays, more than 324 different compounds have been reported worldwide in this cluster of drugs, thus becoming an increasing health problem, particularly in the adolescent population which consumption is substantially greater than the average population (Mathews et al., 2019; Bukke et al., 2021; United Nations Office on Drugs and Crime (UNODC), 2022). JWH-018, HU-210 and AM-2201, among many others, are examples of originally research molecules presently detected in Spice/K2 preparations (Atwood et al., 2010; Funada and Takebayashi-Ohsawa, 2018; Papaseit et al., 2018; Fang and Wang, 2023). These novel drugs are harder than cannabis, since synthetic cannabinoids are potent and efficacious cannabinoid receptor agonists, whereas cannabis preparations contain a variety of different compounds (Cohen and Weinstein, 2018; Hourani and Alexander, 2018). For instance, JWH-018 is a potent agonist at CB1R and CB2R, showing approximately a four-fold increased activity for CB1R and ten-fold affinity at the CB2R, in comparison with THC (Dresen et al., 2010). As a consequence, synthetic cannabinoids have been related to more harmful effects than natural cannabinoids (Ford et al., 2017). Acute intoxication has been linked to seizures, tachycardia, hypertension, anxiety, hallucinations, tachypnea, mydriasis, nausea and vomiting (Davidson et al., 2017). Most alarming, abuse of synthetic cannabinoids has even resulted in death (Trecki et al., 2015).

2. THE OREXIN/HYPOCRETIN SYSTEM

The orexin system, also known as hypocretin system, was concurrently discovered in 1998 by two different research groups: Sakurai et al. (Japan) and de Lecea et al. (United Sates). Sakurai and colleagues identified two novel neuropeptides that strongly activated an orphan receptor (HFGAN72). By sequence homology analysis, a second receptor with the same properties was also characterized. These peptides and the correspondent receptors were termed “orexins” from the Greek word *orexis* meaning appetite, as the central administration of these peptides was shown to stimulate food intake in rats (Sakurai et al., 1998). At the same time, de Lecea and co-workers described a hypothalamus-specific mRNA which encoded the precursor of two similar peptides. Both neuropeptides exhibited substantial amino acid sequence identity with incretins, a group of metabolic hormones related to feeding behaviour. For that reason, they named these peptides “hypocretins” (“hypo-” from hypothalamus; “-cretins” from incretins). Moreover, hypocretins showed an important neuroexcitatory activity in cultured neurons, thus suggesting the role of neurotransmitters in the CNS (de Lecea et al., 1998).

A few months later, it became clear that the orexin and the hypocretin systems were different terms for the same neurotransmission system. Although cDNA accession number of *Mus musculus* prepro-hypocretin (AF019566) was originally different from the one for prepro-orexin (AFO41242), mRNA sequences, chromosomal mapping and brain immunolocalization of both peptides converged towards the same way (Nisoli et al., 1998). Altogether, the names “orexin” and “hypocretin” are currently used as synonyms in the scientific literature. To avoid potential confusion, the term “orexin” will be used throughout the present thesis.

The orexin system was originally known for its physiological role in the central regulation of feeding (Sakurai et al., 1998; Lubkin and Stricker-Krongrad, 1998; Wolf, 1998). However, further investigation also highlighted a pivotal role in the maintenance and control of sleep/wakefulness states and energy homeostasis (Hagan et al., 1999; Lin et al., 1999a; Thannickal et al., 2000a; Mieda et al., 2004a). Nowadays, many other functions associated with the orexinergic system in physiological and pathological conditions have been clearly deciphered (Jacobson et al., 2022).

2.1. Orexins and receptors

Orexin-A (OXA) and orexin-B (OXB) are neuropeptides synthesized by neuronal cells, primarily found in the lateral hypothalamic area (LHA), which comprises the perifornical, lateral, posterior and dorsomedial nuclei (de Lecea et al., 1998; Sakurai et al., 1998; Nambu et al., 1999). These cells express a single-gene localized on chromosome 17q21

(in humans), which encodes the prepro-orexin, a 131-residue polypeptide (Sakurai et al., 1999). By enzymatic cleavage of this common precursor, both orexins are synthesized. OXA is a 33-amino acid peptide with two intramolecular disulphide bridges within the N-terminal, whereas OXB is a linear 28-amino acid peptide (Sakurai et al., 1998). These neuropeptides share 46% homology and have been identified from fish to mammalian species (Wong et al., 2011) (Figure 8). The genetic and molecular organization of orexin peptides amongst vertebrates are highly similar, thus highlighting the strong evolutionary pressure exerted to preserve structural integrity and functions. Indeed, OXA remains highly conserved in the vertebrate species, particularly in mammals with 100% sequence homology, while OXB differs only in 1 or 2 amino acids across species (Wong et al., 2011).

Orexins act on two specific receptors that are widespread distributed across the brain: orexin receptor-1 (OX1R; 425 amino acids) and orexin receptor-2 (OX2R; 444 amino acids) (Sakurai et al., 1998; de Lecea et al., 1998; Peyron et al., 1998). Both receptors belong to the GPCR superfamily with seven transmembrane domains. In humans, OX1R and OX2R are located at chromosomes 1 and 6, respectively, and have 64% amino-acid identity with each other (Kukkonen et al., 2002). Analogously to orexin peptides, orexin receptors remain evolutionary preserved along vertebrate species. Particularly, OX2R is present in most vertebrate lineages, whereas OX1R is specific to mammalian species and evolved only during this latter lineage (Wong et al., 2011). An assay performed to determine the affinity of both orexin peptides towards the correspondent receptors demonstrated that OXA presented similar binding affinity for both receptor subtypes, whereas OXB is ~ 10-fold selective for the OX2R versus the OX1R (Sakurai et al., 1998). In addition, neither OX1R nor OX2R have significant affinity for any other neuropeptide, although they exert some structural similarities to other receptors (Holmqvist et al., 2001) (Figure 8).

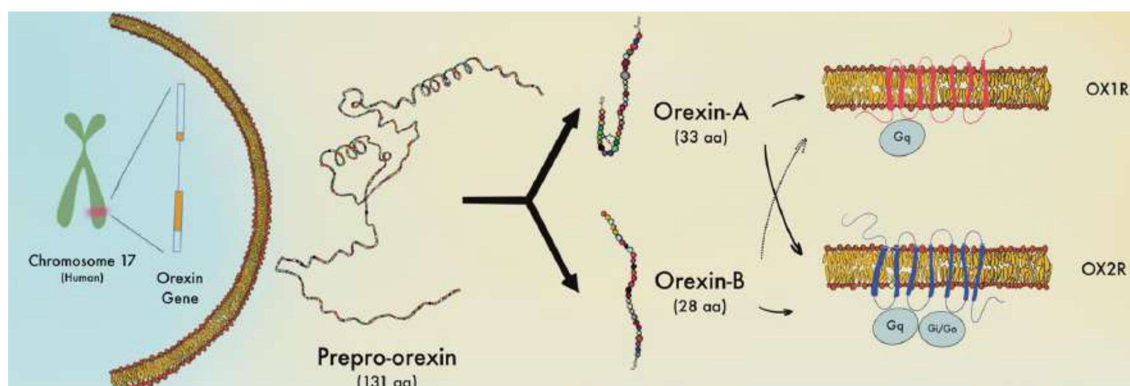

Figure 8. Schematic representation of the orexin peptides biosynthesis.

Orexin signalling is complex and can change depending on the cell type and environment (Kukkonen and Turunen, 2021). As explained before, OX1R and OX2R display the typical intracellular signalling through G-proteins, although only a few studies have focused on this coupling due to technical difficulties (Kukkonen and Leonard, 2014; Kukkonen, 2017). Several studies support that the major primary signalling transducer for orexin receptors is associated with Gq proteins. Nevertheless, this statement is based only on the original finding (Sakurai et al., 1998). Further investigation has also demonstrated a strong coupling to the Gq-mediated responses, consisting of a Ca^{2+} elevation and a phospholipase C (PLC) activation, thus supporting the original hypothesis (Johansson et al., 2007; Putula et al., 2014). In this vein, the G-protein families Gi/o and Gs have been implicated in the orexin signalling (Karteris et al., 2001; Kukkonen, 2016). Downstream signal pathways induce several molecular alterations in neuronal cells. For instance, a neuroexcitatory activity as a consequence of Ca^{2+} increase. This ion elevation is caused by extracellular Ca^{2+} entrance through the modulation of membrane ion channels, as well as Ca^{2+} release from intracellular vesicles (van den Pol et al., 1998; Eriksson et al., 2001). Also, gene expression is modified through the activation or inhibition of protein kinases, mainly including mitogen-activated protein kinases and protein kinase A (Selbach et al., 2010; Guo and Feng, 2012). Finally, arachidonic and phosphatidic acids are also synthesized via phospholipase A2 and D, respectively, downstream the orexin receptor activation (Turunen et al., 2010; Jäntti et al., 2012). These metabolites have a key role in the activation of different cation channels and protein kinases (Jang et al., 2012). The endocannabinoid 2-AG is also synthesized by diacylglycerol hydrolysis following orexin receptor activation (Turunen et al., 2012). As explained in section *1.1.2. Endocannabinoids*, 2-AG is known to be a key regulator of neurotransmitters release and mediates several physiological effects of orexins (Haj-Dahmane and Shen, 2005; Berrendero et al., 2018).

Orexin receptors are also capable of making heteromeric complexes with other GPCRs, at least in recombinant systems (Wang et al., 2019; Raïch et al., 2022). It remains unclear whether this phenomenon happens physiologically. However, if this hypothesis is further confirmed, it might affect orexin signalling, trafficking and pharmacology.

2.2. Neurobiological distribution of the orexin system

Orexin neurons are localized restrictively in the LHA, as described before. Deciphering the projections emitted to other brain areas (i.e. outputs) is essential to elucidate the specific roles of orexins in neuronal and behavioural regulation (Figure 9). However, it is

also relevant to recognize afferent neurons that modulate the activity of the orexin system (i.e. inputs) by releasing either excitatory or inhibitory neurotransmitters onto orexin neurons (Li and de Lecea, 2020).

A study performed with a tracing method of green fluorescent protein (GFP) fused with a nontoxic fragment of tetanus toxin that transfers the tracer in the retrograde direction (Maskos et al., 2002), identified the multiple brain regions which neurons directly make synapse onto orexin neurons (Sakurai et al., 2005a). This study points out the amygdala, basal forebrain, preoptic area, raphe nuclei, ventromedial, dorsomedial and paraventricular nucleus (PVN) in the hypothalamus, septum, infralimbic (IL) and prelimbic (PL) portions of the mPFC, NAc shell and bed nucleus of stria terminalis (BNST), as the main brain regions that modulate the activity of orexin neurons (Sakurai et al., 2005a). In general agreement with these results, a similar pattern of orexin afferent neurons was obtained in further studies by using different tracing techniques (Yoshida et al., 2006; Giardino et al., 2018; Saito et al., 2018).

A few months after the orexin system discovery, Peyron and colleagues illustrated the distribution and relative density of orexin fibres in coronal slices of the rat brain atlas (Peyron et al., 1998). These results were confirmed by latter efforts (Nambu et al., 1999; Date et al., 1999; Mondal et al., 1999; Marcus et al., 2001), which highlighted a broad distribution of the orexin system throughout the whole brain (Figure 9). The different areas receiving orexin projections have been detailed in the next section, according to the most relevant function in which they have been involved.

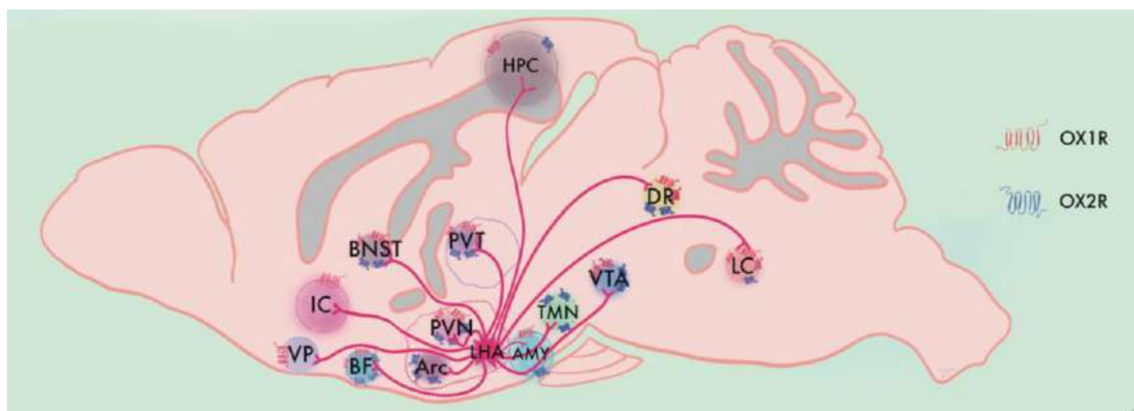

Figure 9. Schematic representation of the neurobiological distribution of the orexin system in the brain. AMY, amygdala; Arc, arcuate nucleus; BF, basal forebrain; BNST, bed nucleus of the stria terminalis; DR, dorsal raphe; HPC, hippocampus; IC, insular cortex; LHA, lateral hypothalamic area; LC, locus coeruleus; OX1R, orexin receptor-1; OX2R, orexin receptor-2; PVN, paraventricular nucleus in the hypothalamus; PVT, paraventricular thalamus; TMN, tuberomammillary nucleus; VP, ventral pallidum; VTA, ventral tegmental area.

2.3. Physiological functions in the CNS

The orexin circuitry and receptor expression profile naturally associate this system with specific clusters of functional roles. These functions include sleep-wake cycle homeostasis, reward and motivation, stress resilience, feeding behaviour and metabolism, and cognition.

2.3.1. Sleep-wake cycle homeostasis

Sleep is defined as a physiological and reversible state of reduced responsiveness to external stimuli and relative inactivity, accompanied by a loss of consciousness. A considerable amount of evidence has established that sleep benefits the retention of memory, as sleep deprivation causes irreversible cognitive dysfunctions (Yang et al., 2012; Hudson et al., 2020; Sabia et al., 2021). Additionally, recent studies have revealed direct and indirect associations between sleep and health risks, such as stroke, neurodegenerative, immunological, endocrine and mood disorders, among others (Liew and Aung, 2021).

Orexin neurons in the LHA project to diverse wakefulness-related brain regions. The locus coeruleus (LC) receives the densest extrahypothalamic projections from orexinergic neurons (Peyron et al., 1998; Date et al., 1999), thus exerting a strong neuroexcitatory activity by releasing orexins, as well as glutamate (Henny et al., 2010). Such region presents abundant OX1R, whereas OX2R expression is lower (Hervieu et al., 2001; Marcus et al., 2001). Orexin projections also achieve serotonergic neurons of the raphe nuclei (Peyron et al., 1998), specifically those neurons localized in the dorsal raphe portion (Lee et al., 2005; Pollak Dorocic et al., 2014). Both orexin receptors are present in this area (Trivedi et al., 1998; Marcus et al., 2001), although OX2R has a more pronounced effect in depolarizing serotonergic neurons of the dorsal raphe (Soffin et al., 2004). Orexin neurons from the LHA send abundant projections to the tuberomammillary nucleus (TMN) (Peyron et al., 1998), which synthesize histamine, a monoaminergic neurotransmitter classically related to sleep-wake cycle regulation (Ramesh et al., 2004; Chu et al., 2004). In that region, OXR2 is much more expressed than OX1R (Marcus et al., 2001; Eriksson et al., 2001), and the neuroexcitatory activity is exerted via direct effect of orexins and glutamate (Bayer et al., 2001; Eriksson et al., 2001), as well as an indirect inhibition of GABAergic projections to the TMN neurons upon dynorphin's suppression (Eriksson et al., 2004). Acetylcholine is another key modulator of the sleep-wake cycle, which neurons are primarily localized in an area at the front and bottom of the brain named basal forebrain (Woolf, 1991). This region includes several nuclei that receive abundant projections from the LHA (Peyron et al., 1998; Villano et al., 2017) and both OX1R and OX2R are equivalently expressed (Marcus et al., 2001). In particular,

cholinergic neurons of the basal forebrain are activated mainly via OX2R in brain preparations (Eggermann et al., 2001). The paraventricular thalamus (PVT) is also involved in the regulation of the sleep-wake cycle. Although this region expresses both orexin receptors (Marcus et al., 2001; Kirouac et al., 2005), OXA and OXB excite postsynaptic neurons in the PVT likely through the activation of OX2R (Ishibashi et al., 2005). Orexins are co-released with dynorphins in these neurons, thus minimalizing orexin's excitatory effect on PVT (Matzeu and Martin-Fardon, 2018).

The first evidence associating the orexin system with the regulation of sleep-wake cycles was reported in 1999, by demonstrating that prepro-orexin or OX2R deficiencies caused narcolepsy in mice (Chemelli et al., 1999) and dogs (Lin et al., 1999), respectively. In agreement, post-mortem brains of narcoleptic patients showed a loss of orexin neurons (Peyron et al., 2000; Thannickal et al., 2000) and reduced orexin concentrations in the cerebrospinal fluid (CSF) of people with narcolepsy (Nishino et al., 2000). Subsequent experiments demonstrated that acute intracerebroventricular (icv) administration of OXA maintained wakefulness, suppressed sleep and avoided cataplectic attacks, in a murine model of narcolepsy (Mieda et al., 2004). Contrary, administration of the OX2R antagonist MK1064 promoted non-rapid eye movement (NREM) and REM sleep across different species (Gotter et al., 2016).

Direct infusion of OXA into the nuclei that receive projections from orexin neurons including the LC (Bourgin et al., 2000), the TMM (Huang et al., 2001) or the basal forebrain (Thakkar et al., 2001) has also been reported to increase the duration of wakefulness and suppressed REM sleep. On the other hand, orexin neurons also receive projections from some nuclei involved in sleep-wake regulation. For example, "sleep-active" neurons of the preoptic area of the hypothalamus contain the inhibitory neurotransmitter GABA and densely project to orexin neurons (Sakurai et al., 2005). Optogenetic stimulation of preoptic area fibres resulted in rapid inhibition of orexin neurons (Saito et al., 2013). On the contrary, orexin neurons are also innervated by cholinergic neurons in the basal forebrain, which have a positive influence on wakefulness. Overall, orexin neurons are inhibited by sleep-promoting neurons and activated by wake-promoting neurons (Shen et al., 2022).

As detailed in section *2.4. Therapeutic agents targeting the orexin system*, preclinical research has paved the road to the discovery and production of novel pharmacological treatments for sleep disorders. In this sense, orexin receptor antagonists have been developed as sleep-promoting agents for patients with insomnia disorder. Orexin agonists could also be developed to alleviate symptoms of narcolepsy and treat

excessive daytime sleepiness and/or hypersomnolence, given the destruction of orexin neurons observed in those patients (Black et al., 2017).

2.3.2. Reward and motivation

The rewarding system normally serves to guide our attention towards and consumption of natural rewards, which entail pleasurable feelings and ensure our survival. This physiological function might also lead to the consumption of drugs of abuse due to their overstated reinforcing properties that, in turn, elicits a dysregulation of the rewarding system. A commonly observed consequence from this overactivity is the onset of psychiatric disorders, such as substance use disorder (Volkow et al., 2019; Maldonado et al., 2021).

The best characterized reward circuit in the CNS involves dopamine neurons in the VTA, which project to the NAc. This is a crucial pathway for the recognition of rewards in the environment and for initiating their consumption. However, dopaminergic neurons in the VTA also innervate other brain regions, including the amygdala, hippocampus, and frontal and limbic cortices, among other areas (Russo and Nestler, 2013). These regions are inter-connected in complex ways and receive several afferent neurons from different neurotransmission systems, which regulate their activity. One of these modulators is the orexin system. Immunohistochemical studies have reported dense orexin projections from the LHA to the VTA, in addition to a large distribution of both OX1R and OX2R in this region in a similar proportion (Peyron et al., 1998; Marcus et al., 2001). Indeed, direct administration of OXA and OXB into the VTA increased dopamine release in mPFC and NAc, thus highlighting the VTA as an important site of action for orexins to mediate rewarding effects (Vittoz et al., 2008). Orexins are able to induce an increased cell firing of dopamine neurons by direct depolarization, as well as an indirect effect through the interaction with other neurotransmitters within the VTA (Korotkova et al., 2003; Borgland et al., 2008). Another reward-related structure is the ventral pallidum, which neurons receive dense projections from orexin neurons with the particularity that only OX1R is expressed in this structure (Peyron et al., 1998; Ch’ng and Lawrence, 2015). A study published by Ji and colleagues demonstrated that orexins directly activate GABAergic neurons in the ventral pallidum to achieve higher hedonic tone and prevent depressive-like behaviour (Ji et al., 2019). Finally, the insular cortex, a less understood region in brain functional knowledge that mainly expresses OX1R, has also been related to rewarding properties in addiction experiments (Marcus et al., 2001; Hollander et al., 2008).

Rewarding properties of several compounds are regulated by the orexin system. Hence, orexins induce food intake not only as a consequence of metabolic requirements, but also as a physiological response to hedonic feeding (Saper et al., 2002), as explained below (see section *2.3.4. Feeding behaviour and metabolism*). Similarly, orexins also modulate the addictive-associated behaviour caused by the most common consumed drugs of abuse, including cocaine, opioids, alcohol, nicotine, and cannabinoids (McGregor et al., 2021). For instance, OX1R antagonism decreased operant responding for cocaine in discrete trials with limited infusions per hour (España et al., 2010), in protocols with extended access to cocaine (Schmeichel et al., 2017), and in rats that displayed an addicted-like phenotype (James et al., 2019). Conversely, infusion of OXA or OXB in the PVT increased alcohol intake in an intermittent access procedure, an effect that was reversed by OX2R blockade (Barson et al., 2015, 2017). These are only a few examples demonstrating the involvement of the orexin system in the neural mechanisms mediating the rewarding and addictive properties of several drugs of abuse. For that reason, normalizing the orexin function with novel therapeutic approaches may offer the opportunity to improve the clinical management of drug addiction (Mehr et al., 2021). Indeed, the National Institute of Drug Abuse has recognized orexin antagonist and/or negative allosteric modulators among their list of priority targets for new medications to cope with the opioid crisis (Rasmussen et al., 2019).

2.3.3. Stress resilience

Stress can be defined as the state of disturbed homeostasis that occurs in case of real or perceived threat (Misiak et al., 2020). This physiological reaction may induce a maladaptive response when it is repeatedly presented over a considerable period of time. Convincing evidence indicates that stress has a key role in the aetiology of several mental diseases, including mood (i.e., major depressive and bipolar disorders), anxiety, and fear-related disorders (i.e., posttraumatic stress disorder (PTSD), panic, and phobias) (Smoller, 2016; Barbano et al., 2019). The orexin system is a major modulator of the stress response, since the most relevant stress-sensitive brain areas receive dense hypothalamic projections from orexin neurons (Peyron et al., 1998). On the one hand, the amygdala involves diverse nuclei that mainly express OX1R, except for the lateral amygdala in which OX2R is much more abundant than OX1R (Marcus et al., 2001). Importantly, the amygdala also receives indirect orexin projections through noradrenergic neurons from the LC (Sears et al., 2013; Soya et al., 2017) and serotonergic neurons from the dorsal raphe (Hasegawa et al., 2017). Another important region regulating stress is the PVN, which presents an abundant expression of both

orexin receptors, although OX2R has a prominent role in this nucleus (Marcus et al., 2001; Cluderay et al., 2002).

Clinical and preclinical studies clearly differentiate between acute and repeated stress, given the pathological consequences resulting from the second type. Indeed, several studies have described the involvement of the orexin system in acute stress reactions, whereas less is known about the role of orexins in chronic stress.

2.3.3.1. Acute stress

The acute stress response is a spontaneous and natural reaction against potential threats and unpredictable events. It prepares the body to cope in a survival situation by increasing arousal through the hypothalamic-pituitary-adrenal (HPA) axis activation, among other mechanisms (Sargin, 2019). Central administration of orexins induces an acute stress reaction which produces grooming, burrowing and face washing in rats (Ida et al., 1999). Accordingly, orexin deficient mice presented the opposite effect by reducing the activity in a resident-intruder paradigm, thus indicating a diminished behavioural response to stress (Kayaba et al., 2003). An interesting study recently published by Yaeger and co-workers indicates that both antagonism or genetic knockdown of OX1R in the basolateral amygdala (BLA) switches the behavioural expression in the Stress Alternatives Model. In this test, smaller mice are placed with a larger novel aggressor and given the opportunity to exit through escape tunnels. Blockade or downregulation of OX1R induced a stress-resilient response (i.e. escape), in comparison to the control mice that presented a stress-sensitive response (i.e. stay) (Yaeger et al., 2022). Reciprocally, acute stress augments orexin neuron activity. In this sense, a single restraint in rats increased the percentage of active orexin neurons marked with cFos, an indicator of neuronal activity (Grafe et al., 2017).

The molecular basis underlying these alterations mainly relies on the HPA axis and the sympathetic nervous system, which are essential components to initiate stress-coping mechanisms. On the one hand, orexins favour HPA axis response to acute stress at all levels, as well as HPA activation promotes orexin activity. For example, icv infusion of orexins activates corticotropin-releasing factor (CRF)-expressing neurons and increases downstream HPA hormones: adrenocorticotropic hormone (ACTH) and corticosterone (Kuru et al., 2000; Al-Barazanji et al., 2001). Moreover, ACTH increased levels are mediated by OX1R and OX2R in the pituitary (Date et al., 2000). Orexins also act peripherally on the adrenal glands, thus stimulating glucocorticoid secretion (Mazzocchi et al., 2001). In the opposite direction, activation of the HPA axis by CRF administration increases orexin neuron activity, as revealed by electrophysiological data of mice slices

(Winsky-Sommerer et al., 2004). On the other hand, sympathetic nervous system activation is acutely induced by orexins. Sympathetic activity signs, such as blood pressure, heart rate, and renal sympathetic nerve activity are significantly increased by orexin central administration in rats (Shirasaka et al., 1999). Accordingly, orexin knockout (KO) mice exhibited attenuated heart rate and blood pressure in the resident-intruder test (Kayaba et al., 2003).

2.3.3.2. Repeated stress

The role of the orexin system on the modulation of repeated stress response remains to be clearly defined. While acute stress positively and reciprocally correlates with orexin activation, the role of the orexin system in repeated stress depends on several factors such as the intensity, type, and duration of the stressor. Also, the ability to habituate to the stressful stimuli is a key element to determine the activity of the orexin system. For example, repeated restraint in male rats for five days (30 min per day) decreased both OXA levels in the CSF and orexin neuronal activation (Grafe et al., 2017). Conversely, 14 days of repeated restraint (2 h per day) in male mice produced the opposite effect, an increase of orexin mRNA transcripts in the BLA (Kim et al., 2015). Taking this comparison into account, a stronger-intensity and longer-duration stressor may probably cause a lack of habituation which would explain the increased orexin activity. In another study, unpredictable chronic mild stress (UCMS; different stressors randomly presented) in male mice produced a significant activation of orexin neurons in the dorsomedial and perifornical hypothalamic area (Nollet et al., 2011). Therefore, variations in the type of stressor prevent habituation which, in turn, increases orexin activity. In conclusion, the intensity, duration, and type of stressor stimuli are critical parameters to induce or prevent habituation in a repeated stress paradigm, which seems to negatively correlate with orexin activity.

A dysregulation of the orexin system has been observed in several stress-related neuropsychiatric illnesses, including mood, anxiety, and fear-related disorders. In this sense, mood disorders are linked to a hypofunction of the orexin system, although several studies on the opposite direction have also been published. For instance, house isolation, a commonly used stressor, induced anhedonia-like symptoms in the resident-intruder social defeat paradigm. These animals displayed reduced levels of both orexin peptides in the hypothalamus, mPFC and VTA (Nocjar et al., 2012). Another study revealed that OXA administration exerted antidepressant-like effect in mice that underwent chronic social defeat stress (Chung et al., 2014). In contrast, UCMS-induced depressive-like behaviour in male mice showed increased orexin neuron activity. In the

same study, chronic administration of the DORA almorexant promoted antidepressant-like effects in the tail suspension test (Nollet et al., 2011). In human studies, plasma OXA levels of both unipolar and bipolar depressive patients are decreased (Ünler et al., 2022), as well as CSF levels of OXA in depressive suicidal patients (Brundin et al., 2007). However, another clinical study has shown increased OXA plasma levels in major deressive and bipolar disorder patients (Li et al., 2021).

Clearer results have been obtained in studies evaluating anxiety and fear-related behaviours. A key study by Suzuki and co-workers reported an anxiety-like behaviour in the elevated plus maze and light-dark tests after central administration of OXA in male mice and rats (Suzuki et al., 2005). In the same vein, cat odor-induced anxiety in rats was attenuated by the OX1R antagonist SB-334867. This anxiogenic effect induced an overactivation of some stress-related brain areas, such as the PVN and dorsal premammilary nucleus, which was reduced by OX1R blockade (Vanderhaven et al., 2015). A clinical investigation with adolescents aged 12-18 years indicated that serum OXA levels were significantly higher in adolescents with anxiety disorders in comparison with healthy individuals (Akça et al., 2020). Similarly, a strong positive correlation has been extensively reported between the severity of fear-related diseases and the orexin system activation. For example, icv infusion of OXA impaired fear extinction in mice in both contextual and cued tests, while the OX1R antagonist SB-334867 facilitated this response (Flores et al., 2014). In agreement, the activity of orexin neurons was negatively correlated with successful extinction of conditioned fear in rats (Sharko et al., 2017). The underlying mechanisms of the orexin system modulating fear extinction have been widely addressed in the present thesis (Article 2).

2.3.4. Feeding behaviour and metabolism

Orexins were originally known to modulate feeding behaviour since central administration in rats induced food intake (Sakurai et al., 1998). Further research has replicated this effect in different animal species, even in zebrafish (Yokobori et al., 2011). The main orexin projections-receiving structure involved in feeding and body-weight regulation is the arcuate nucleus of the hypothalamus, which neurons mainly express OX2R (Marcus et al., 2001). Indeed, microinjection of OXA into such region produced a significant activation of neuropeptide Y-expressing neurons, which reciprocally connect with LHA orexin neurons (Muroya et al., 2004; Fu et al., 2004). Also, BNST modulates feeding behaviour, among many other functions, like reward and arousal. This region receives dense projections from the LHA and both orexin receptors are expressed, although OX1R is more prominent (Peyron et al., 1998; Marcus et al., 2001).

Preclinical studies have made important progress investigating the role of the orexin system in the regulation of feeding behaviour and metabolic processes. In this sense, an induced hypofunction of the orexin system by OX1R antagonism or anti-orexin antibody attenuated food consumption (Haynes et al., 2000; Yamada et al., 2000). Given the key role of the orexin system in reward and motivation, orexins also stimulate hedonic feeding. Hence, chronic palatable food exposure activated orexin neurons by enhancing orexin signalling to several brain output areas in rodents. In mice exposed to a high-fat diet, changes in orexin neuronal activity have been found (Horvath and Gao, 2005) and different studies report increased prepro-orexin mRNA levels in rats and monkeys exposed to a high-fat diet also during early life (Beck et al., 2006; True et al., 2018). Additionally, OX1R antagonism not only altered operant seeking behaviour for standard food in food-restricted mice (Sharf et al., 2010), but also reduced both motivational and primary reinforcement in rats trained to seek for high-palatable food, even under satiation (Choi et al., 2010). These findings support the hypothesis that the orexin system is involved in compulsive eating, food-seeking behaviour, and food craving associated with the failure of homeostatic control of food consumption (Pich and Melotto, 2014). For that reason, diverse clinical and preclinical studies have reported orexin implications in the psychopathology and treatment of eating disorders, such as bulimia nervosa, anorexia nervosa, and binge eating disorder (Janas-Kozik et al., 2011; Piccoli et al., 2012; Sauchelli et al., 2016).

The orexin system also monitors humoral and neural indicators of energy balance. Indeed, activity of orexinergic cells is inhibited by increased levels of glucose and leptin, a hormone mainly released from the adipose tissue which attenuates food consumption and increases body energy expenditure (Yamanaka et al., 2003; Burdakov et al., 2005). Conversely, a hypoglycaemic state or reduced levels of ghrelin, a gastric-derived hormone that increases the sensation of hunger, activate orexin neurons in the LHA (Briski and Sylvester, 2001; Toshinai et al., 2003). In accordance with these results, different studies have reported a significant increase of prepro-orexin and orexin receptors mRNA levels after fasting in rats (Cai et al., 1999; Lu et al., 2000). Therefore, orexin signalling is modulated according to the energetic requirements, to achieve a proper metabolic response.

2.3.5. Cognition

Recent research has determined a role for orexins in cognitive processes, in line with orexin projections and expression of both receptors in cognitive-related brain areas, essentially the hippocampus. This complex structure expresses both OX1R, mainly in

the dentate gyrus and CA1, and OX2R in CA3 (Elahdadi Salmani et al., 2022). Also, medial and lateral septum play a secondary role in cognitive processes, thus presenting considerable levels of OX2R (Marcus et al., 2001). Therefore, preclinical studies with mice have demonstrated an involvement of the orexin system in hippocampal-dependent social and spatial memory (Yang et al., 2013; Aitta-aho et al., 2016; Mavanji et al., 2017), as well as a regulatory role in cognitive flexibility, in a sex-specific manner (Durairaja and Fendt, 2021). Moreover, intranasal OXA administration improved age-related cognitive dysfunctions in a rodent model of aging (Calva et al., 2020). These data reveal the potential benefits of targeting the orexin system for cognitive-related disorders.

Neurodegenerative diseases presenting severe cognitive disruption, such as AD or PD, have been associated with orexin dysregulations. Some reports addressing AD showed increased CSF orexin levels (Liguori et al., 2014; Gabelle et al., 2017), whereas others sustain that orexin levels remain within the normal range or below (Fronczek et al., 2012; Slats et al., 2012). However, normalizing orexin levels through the activation of cholinergic neurons, which are critical for cognitive function and remain degenerated in AD (Shekari and Fahnestock, 2021), improved attentional processing and ameliorated cognitive decline (Zajo et al., 2016; Erichsen et al., 2021). Another mechanism that partially explains cognitive deficiencies in AD are sleep disturbances (Friedman et al., 2007; Liguori et al., 2016). Consistently, knocking out the orexin gene in a transgenic AD mouse model decreased the formation of Aβ plaques and attenuated sleep fragmentation, whereas rescuing orexinergic neurons increased wakefulness and induced Aβ accumulation (Roh et al., 2014).

Patients with PD showed less orexin immunoreactive neurons compared to matched controls (Fronczek et al., 2007; Thannickal et al., 2007). Further research confirmed that orexin levels were inversely correlated with disease severity and progression, as well as with cognitive dysfunction (Asai et al., 2009; Huang et al., 2021). Indeed, preclinical studies have used transgenic mouse models of PD, such as A53T mice, which displays early social cognitive alterations and hippocampus-dependent memory impairment (Paumier et al., 2013). Chemogenetic activation of orexin neurons reversed alterations in sociability in this mouse PD model, as well as spatial and object recognition memory (Stanojlovic et al., 2019). Thus, loss of orexin neurons and hypofunctional orexinergic transmission occur in PD, contributing to the cognitive impairments of the disease. Orexin replacement therapy could potentially attenuate cognitive clinical manifestations of PD and delay disease progression.

2.4. Therapeutic agents targeting the orexin system

Since its discovery, the orexin system has become one of the most profoundly studied neuroregulatory systems. Cumulative research in this field has given rise to a wide variety of physiological functions in which orexins are described to be involved, as mentioned above. For that reason, pharmaceutical companies have been encouraged to develop more than 140 clinical trials to date, by testing diverse orexin-targeting candidates for different pathological conditions. Sleep/wake disorders are the most studied, although many others like substance use disorders, mood and anxiety disorders, and cognitive impairments are also addressed by modulating the orexin system. These efforts entail an important economic burden, thus highlighting the increased expectation for the orexin system to become a suitable target for many non-well treated disorders such as insomnia and narcolepsy.

2.4.1. Orexin receptor antagonists

At least, 50 OX1R and/or OX2R selective antagonists have been characterized (Perrey and Zhang, 2020). They are broadly divided into single orexin receptor antagonists (SORAs) and dual orexin receptor antagonists (DORAs).

The first SORA was SB-334867, an OX1R-selective antagonist initially tested for sleep and feeding dysregulations (Rodgers et al., 2001; Smith et al., 2003). Chronic insomnia is defined as difficulty initiating and/or maintaining sleep on at least 3 nights per week for at least 3 months (Ohayon, 2002). Currently, benzodiazepines are the most commonly prescribed drugs for the treatment of this condition (Riemann et al., 2017). These compounds are positive allosteric modulators of the $GABA_A$ receptors, thus inhibiting neuronal activity. Despite their efficacy, benzodiazepines present important side effects such as residual sedation throughout the day, memory impairment, and abuse and physical dependence (Edinoff et al., 2021). As orexins play an important role in the maintenance of arousal, orexin receptor antagonism has been purposed as a novel alternative strategy to treat insomnia. However, SB-334867 failed to obtain convincing results, as one of the main issues of OX1R antagonism is the nonselective binding to other receptors like adenosine and serotonin receptors. OX2R antagonists such as MK-1064 present major specificity than OX1R antagonists (Roecker et al., 2014). Nevertheless, effectiveness of SORAs is substantially decreased when compared with DORAs. For that reason, most of the current clinical trials addressing the orexin system are based on the development of DORAs. Daridorexant and Suvorexant, both DORAs, are the only orexin-targeting drugs recently approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) in Europe and the United

States, respectively. Lamborexant, another DORA, has been licensed only by the FDA (Subramanian and Ravichandran, 2022). These drugs are currently used to treat sleep latency and sleep maintenance in insomnia cases.

Recent and increasing evidence suggests that orexins could also play an important role in the pathophysiology of several tumors, as orexin receptors have been detected in different types of neoplasms. Specially, in gastrointestinal tumors, but also cervix, prostate, kidney, adipose tissue, testicles, and hematological malignances (e.g., lymphomas). A positive correlation has been found between orexin receptors and the progression of cancer, thus indicating that orexin antagonism might have a potential therapeutic effect (Couvineau et al., 2018, 2022; Alain et al., 2021).

2.4.2. Orexin receptor agonists

Many orexin receptor antagonists have been so far synthesized and, conversely, only a few non-peptide orexin receptor agonists are currently available (Nagahara et al., 2015; Hong et al., 2021). Orexin receptor agonism has emerged as a promising therapeutic avenue for the treatment of diverse pathologies, including narcolepsy and other disorders with excessive daytime sleepiness. Currently, these pathologies are mainly treated with systemic psychostimulants like modafinil or dexamphetamine, which produce severe side effects such as arrhythmias, high blood pressure, insomnia, and weight loss, among others. An interesting strategy for such disorders is the orexin replacement therapy. In human studies, OXA has been intranasal administered to reverse orexin neurons deficiency, thus inducing an arousal state. However, these therapies have not demonstrated efficacy, most likely due to insufficient brain exposure. Other administration routes already studied in animal models of narcolepsy, such as intrathecal injection, may be a viable option which requires further investigation in humans. On the other hand, non-peptide low molecular weight, bioavailable and brain penetrant OX2R agonists are appealing due to the main role of OX2R in wake-promoting states. Promising candidates like TAK-925 are currently tested in early phases of clinical trials with healthy volunteers and individuals with narcolepsy (Evans et al., 2022). An increased wakefulness with the absence of adverse effects would strongly convert this candidate into a suitable therapy to treat narcolepsy and other excessive daytime sleepiness disorders.

2.5. Interaction between the orexin and the endocannabinoid systems

The orexin and the endocannabinoid systems are concomitantly expressed in diverse regions throughout the CNS (e.g., amygdala, hypothalamus, raphe nuclei, LC), thus sharing physiological functions (Maldonado et al., 2006; Wittmann et al., 2007; Tsujino

and Sakurai, 2009; Häring et al., 2015). In accordance with the overlapping distribution in concrete brain areas, electron microscopy studies with Chinese hamster ovary cells revealed the presence of CB1R-OX1R heterodimers (Hilairet et al., 2003), which was further confirmed by in-vitro experiments in different cell types (Ellis et al., 2006; Ward et al., 2011), and in ex-vivo studies with embryonic mouse hypothalamic neurons (Imperatore et al., 2016). Likewise, OX2R was demonstrated to be capable of forming constitutive heteromeric complexes with CB1R, by using a bioluminescence energy transfer (BRET) assay (Jäntti et al., 2014). A recent investigation with microglial cells from animal models of AD has also shown the ability of CB2R to interact with OX1R in order to generate functional heterodimers (Raïch et al., 2022). Hence, heteromerization of orexin with cannabinoid receptors might give rise to the optimization of current and new pharmacological strategies.

Besides receptors coupling, the orexin and the endocannabinoid systems are tightly linked through the downstream molecular signalling pathway after the activation of GPCR orexin receptors (Berrendero et al., 2018). Specifically, Gq-dependent activation of PLC produces diacylglycerol, which in turn is used as a substrate by the membrane-associated enzyme DAGL to produce the endocannabinoid 2-AG (Turunen et al., 2012). A recent study with obese and high-fat content diet mice observed increased OXA and 2-AG levels in these groups, compared with control mice. Accordingly, blockade of OX1R with SB-334867 diminished 2-AG levels, thus improving biochemical alterations associated with the obese phenotype (Forte et al., 2021). A similar discovery associating the orexin and the endocannabinoid systems is the role that the orexin system plays in the regulation of compulsive reward-seeking behaviour in obese mice, thus modulating dopaminergic transmission through 2-AG/CB1R-mediated retrograde signalling (Tunisi et al., 2021). These biochemical interactions entail a cross-modulation of the main functions exerted by the orexin and the endocannabinoid systems, as detailed hereunder.

A growing body of evidence indicates a functional cross-talk between the orexin and the endocannabinoid systems. In this respect, the endocannabinoid system has been shown to be involved in the effects of orexins, mainly in food intake and energy balance, rewarding and motivation, and nociception (Berrendero et al., 2018). For that purpose, 2-AG can retrogradely modulate the release of neurotransmitters after the activation of orexin receptors (PLC-DAGL pathway), as previously explained. Moreover, CB1R located in presynaptic terminals might also regulate neurotransmission release that further activates/inhibits orexinergic neurons. For instance, an interesting study

demonstrated that orexigenic effect induced by OXA was blocked through the peripheral administration of the CB1R antagonist rimonabant (Crespo et al., 2008). The endocannabinoid system might regulate food intake by modulating orexin activity, since administration of the CB1R inverse agonist AM251 decreased OXA immunoreactivity in the hypothalamus, and significantly reduced food intake (Merroun et al., 2015). Concerning nociception, a key study revealed that OX1R activation in the periaqueductal gray (PAG; a midbrain region crucial for initiating descending pain inhibition) slices increased 2-AG synthesis, thus inhibiting GABAergic tone, and increasing PAG neuronal activity (Ho et al., 2011). Finally, a comprehensive study with male mice demonstrated a common molecular mechanism between the orexin and the endocannabinoid systems in drug addiction. In this case, stress-induced cocaine relapse entailed a clear activation of orexin neurons in the LH, and increased OXA levels in the VTA. Consequently, 2-AG was synthesized via PLC-DAGL pathway, and retrogradely inhibited GABA release by CB1R activation. As a result, inhibition of the inhibitory tone evoked an overactivation of VTA dopaminergic neurons, thus increasing rewarding properties and the subsequent cocaine relapse (Tung et al., 2016). These are only a few examples of the increasing evidence demonstrating a biochemical and functional interaction between the endocannabinoid and the orexin systems (Figure 10). However, this interaction remains to be elucidated in the case of fear behaviour, thus becoming one of the main objectives in the present thesis (Article 2).

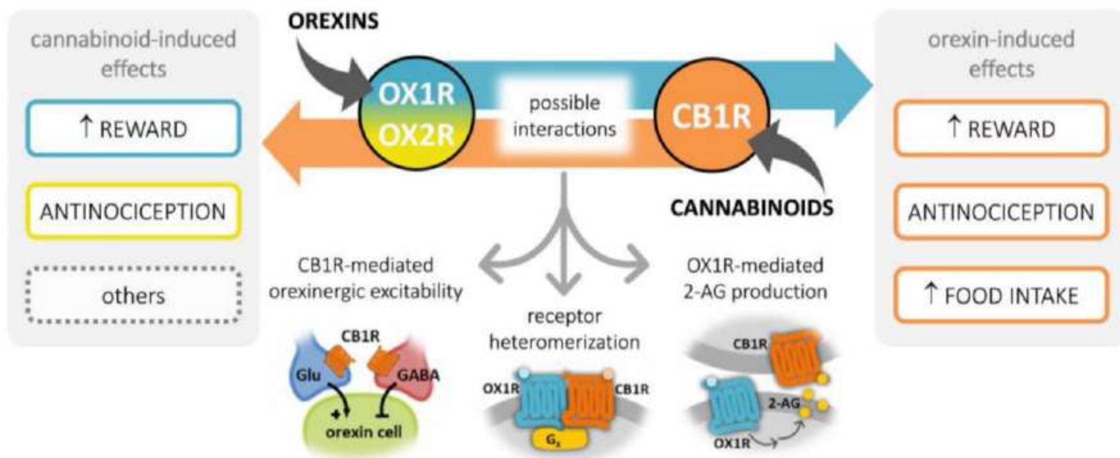

Figure 10. Endocannabinoid and orexin systems molecular interactions. (Adapted from Berrendero *et al*, 2018).

3. FEAR RESPONSE

Fear entails an evolutionary-preserved emotional response, which allows individuals to recognise threatening situations in order to avoid or reduce damage and ensure its survival. This defensive response is present in many animal species, thus highlighting the crucial relevance of coping with dangerous environments (Tovote et al., 2015). A group of behavioural, hormonal, autonomic and physiological reactions underly such response to properly react against potential threats. For instance, the behavioural natural response to fear in animal models is usually referred to as freezing behaviour, a measurable parameter in the Pavloavian fear conditioning paradigm consisting of a complete absence of movement, except for breathing (Blanchard and Blanchard, 2008; Flores et al., 2014). Moreover, other parameters have also been related to fear behaviour, such as the startle response, which is significantly increased under fear dysregulations in preclinical and human studies (Daldrup et al., 2015; Li et al., 2022). Another profoundly described mechanism is the HPA axis overactivity and the subsequent upregulation of its components (e.g., CRF receptors), which has been described as a consequence of fear activation in both animal models and humans (Heitland et al., 2016; Dunlop and Wong, 2019). These are some examples of the wide variety of underlying processes activated under threating conditions that evoke a fear response.

Conceptually, fear can be classified as innate or learned, depending on the stimuli nature that triggers the aversive behaviour. Innate fear is a nonacquired response, which is natural to each animal species. Examples of innate fears include heights, fast approaching objects, pain or predators, among others. Avoid predatory detection is decisive for most animal species survival, although such threat is not common in humans. Analogously, we are usually challenged by social hazards like angry or fearful facial expressions. On the other hand, learned fears are captured throughout life for the exposure to harmful and traumatic situations (Ren and Tao, 2020).

Once fear is acquired and consolidated, diverse neural circuits and their molecular mediators are activated to retain such emotional memory and avoid this threat in the future. However, a dysregulation of aversive memories may lead to detrimental effects, such as the inability to extinguish fear memories and the persistent somatization of this response. These pathological consequences are experimentally addressed by using different paradigms in humans and animal models, thus identifying new potential targets to develop accurate and effective treatments for these neuropsychiatric disorders.

3.1. Clinical classification of fear-related disorders

Fear memories have been found to be pathologically retained in tow different cluster of psychiatric diseases in the DSM-5: trauma- and stressor-related disorders, and anxiety disorders (Figure 12). The first group includes mental illnesses in which diagnostic criterion explicitly requires exposure to an extreme stressor, such as sexual violence, severe injury, or life-threatening situations, by way of direct confrontation or witnessing (American Psychiatric Association, 2013). More Specifically, PTSD is a frequent psychopathology with a considerable lifetime prevalence (~10%) that has been recurrently addressed in basic and clinical studies. This mental disorder entails a temporary difficulty to accurately differentiate between safety and danger, and a frequent struggle to suppress fear in the presence of safety cues (Friedman et al., 2011; Williamson et al., 2021). Symptoms must persist at least one month (otherwise, it is considered acute stress disorder) and are classified in four different clusters: intrusive memories, avoidance of distressing memories, disturbed emotional states, and alterations of arousal and reactivity (Jorge, 2015) (Figure 11A). The presence of these symptoms is variable among individuals with PTSD. However, they end up causing a predominant inability of the daily tasks, (i.e., social and occupational functioning) and a comorbidity with other diseases, such as sleep disturbances, cardiovascular alterations, and substance use disorders, among others (Miller et al., 2017; Back and Jones, 2018; Kondev et al., 2021; O'Donnell et al., 2021) (Figure 11B).

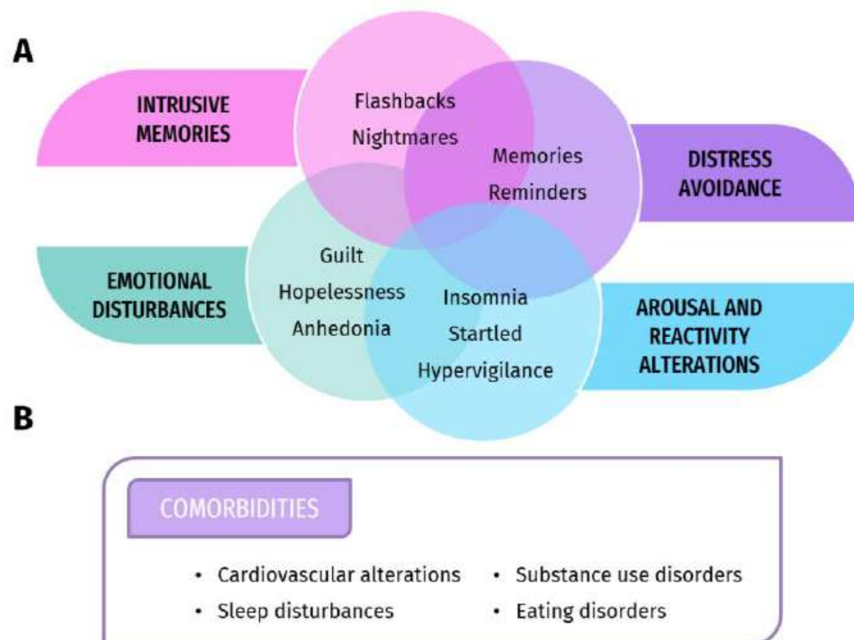

Figure 11. Clinical traits of posttraumatic stress disorder. (A) Venn diagram illustrating the 4 main clusters of symptoms and the interactions among them. (B) Common pathologies associated to posttraumatic stress disorder.

Presenting similar clinical traits with the previous group of psychiatric diseases, anxiety disorders share features of excessive fear and anxiety, and related behavioural disturbances (Figure 12). Anxiety can be defined as the anticipation of future threat, characterized by a vigilance preparation state to detect future danger, and cautious or avoidant behaviour. However, some of the disorders listed in this group present fearful states: phobias (specific phobia, social phobia and agoraphobia) and panic disorders (American Psychiatric Association, 2013). Phobias are characterized by an excessive fear to an object or situation for more than six months, which impossibility to avoid it cause significant distress, tachypnoea, dizziness and feelings of pain, among many other symptoms. Specific phobias involve an oversized fear to particular objects or situations, like spiders, snakes, needles, blood or flying (American Psychiatric Association, 2013; Eaton et al., 2018). Individuals with social phobia, also termed as social anxiety disorder, present marked fear to be judged by other people when interacting with them, being observed or performing in front of others (American Psychiatric Association, 2013; Leichsenring and Leweke, 2017). Finally, agoraphobia is related to a perceived difficulty or inability to escape from situations, such as public transportation, open or enclosed spaces, a surrounding crowd and being outside of the home alone (American Psychiatric Association, 2013; Asmundson et al., 2014). On the other hand, panic disorder is defined by recurrent and unexpected panic attacks that occur without a clear reason. These attacks are characterized by the rapid onset of severe fear with at least four of the physical and psychological symptoms listed in the DSM-5, including palpitations, sweating, trembling and chest pain, among others (American Psychiatric Association, 2013; Locke et al., 2015).

In summary, excessive fear is a core process in PTSD, phobias and panic (Figure 12), although other psychiatric disorders may also present episodes of fear dysregulation at some stage. Despite this common clinical feature, the aetiology, diagnostic and symptoms are significantly different between them (American Psychiatric Association, 2013). However, an effective pharmacological treatment for these fear-based psychopathologies is still distressingly underdeveloped. Symptomatic treatment (e.g., antidepressants or anxiolytics), in addition to psychotherapy and breathing exercises, are the only solution to attenuate the effects of these mental disorders (Kim et al., 2013; Charney et al., 2018; Garakani et al., 2020; Ziffra, 2021). Hence, elucidating the neurobiological mechanisms regulating fear response has become a must in neuroscience research to develop new and effective pharmacological strategies for fear-related disorders, and constitutes the main objective of this thesis.

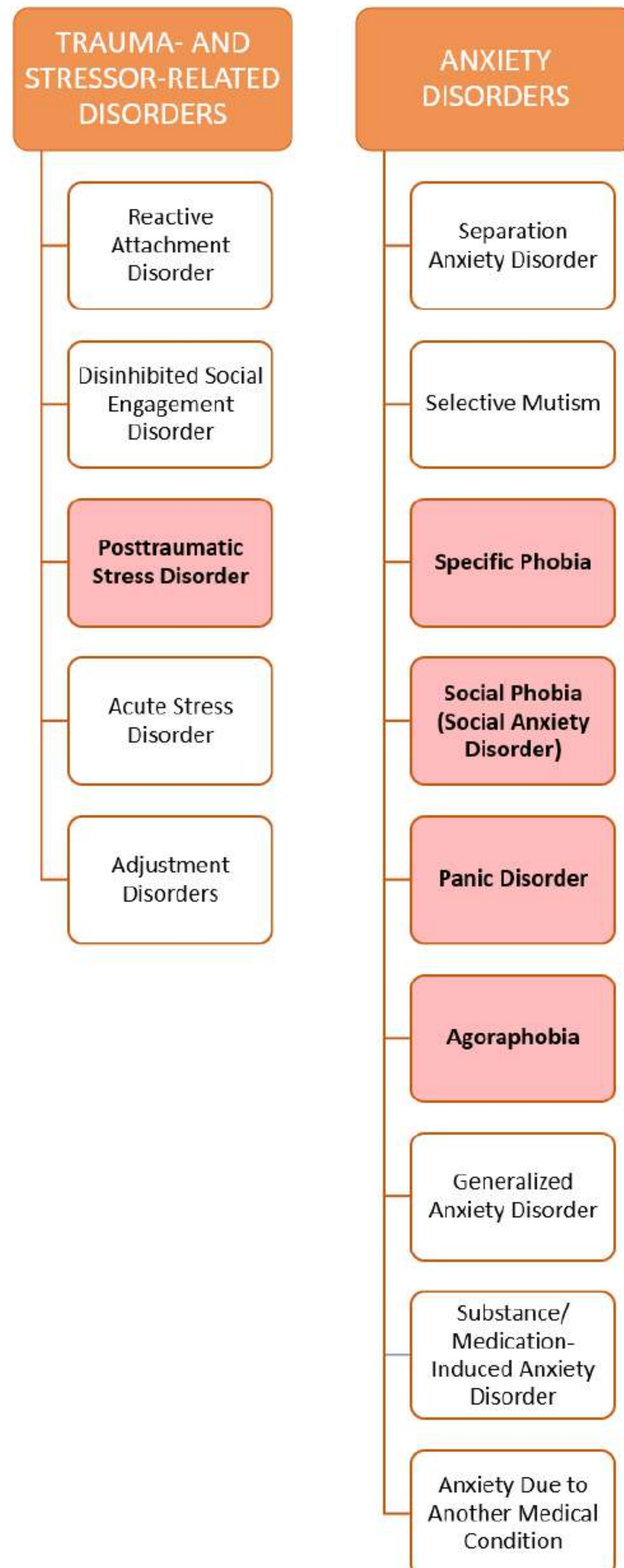

Figure 12. Clinical classification of trauma- and stressor-related disorders, and anxiety disorders based on DSM-5. Fear-related disorders are represented in red-coloured boxes.

3.2. Neurobiological substrates of fear learning and memory

Understanding brain circuitry and the underlying molecular substrates regulating fear, allows us to find successful targets which modulation might become an effective treatment for fear-related disorders. As neural circuits responsible for defensive responses have been substantially preserved across evolution, most of the results and current knowledge is inferred from studies conducted with animal models, mainly rodent species (Namkung et al., 2022).

3.2.1. Neuroanatomy of fear learning and memory

Much research has been devoted to clearly elucidate the brain structures responsible for regulating fear behaviour. Although such a complex response requires many players, the three classical areas involved in fear learning and memory are the amygdala, the mPFC, and the hippocampus (Sierra-Mercado et al., 2011). In order to better clarify the role of fear circuitry, it is important to understand the fear conditioning test primarily used in preclinical research, as explained below (See section *3.3.1. Experimental approaches of fear evaluation*). Briefly, classical (Pavlovian) fear conditioning is based on a strong associative learning process in which a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US). After this association (i.e., fear conditioning), re-exposure to CS alone entails a fear response that will progressively decrease, since the animal starts a dissociative learning by decoupling CS from US (i.e., fear extinction) (Pavlov, 1927; Estes and Skinner, 1941) (Figure 14).

Fear learning or conditioning is mainly conducted by the amygdala. This multiple nuclei-composed area receives several inputs carrying the CS and US information, thus integrating them and orchestrating a fear response through the different projections to central and peripheral structures. Specifically, BLA is a subdivision of this area that includes lateral, basal, and accessory basal nuclei (LeDoux, 2007). The lateral amygdala receives the auditory sensory information (i.e., CS) via two excitatory glutamatergic pathways: a direct projection from the medial geniculate nucleus and the adjacent posterior intralaminar nucleus of the thalamus, and an indirect pathway arising from the auditory thalamus that relays the information via the auditory cortex (McCabe et al., 1993; Campeau and Davis, 1995; Luchkina and Bolshakov, 2019). While auditory information of a simple CS, such as a pure tone, might be conveyed with either pathway, complex auditory stimuli require at least the thalamo-cortico-amygdala pathway (Romanski and LeDoux, 1992; Boatman and Kim, 2006). Conversely, the hippocampus assembles the contextual inputs to generate a single representation of the whole environment, thus sending this information to the basal and accessory basal amygdala

(Maren and Holt, 2000; Bissiere et al., 2011; Chaaya et al., 2018). In a simpler way, the aversive information (i.e., US) is transmitted to the lateral amygdala from the somatosensory thalamus and somatosensory cortex (Shi and Cassell, 1998; Lanuza et al., 2004). However, some other mechanisms have been postulated depending on the stimulus nature (Shi and Davis, 1999; Orsini and Maren, 2012; Herry and Johansen, 2014). Once both stimuli reach the mentioned nuclei of the amygdala, these signals converge into the BLA by generating lasting synaptic enhancements in the CS inputs (Romanski et al., 1993; Luchkina and Bolshakov, 2019). Also, projections from PL (dorsal anterior cingulate cortex in humans) to the BLA are necessary in this process to properly generate learned fear memories (Corcoran and Quirk, 2007; Chen et al., 2022). It is at this point that synaptic plasticity plays a key role to correctly encode conditioned fear memory as a result of the US-CS association (Luchkina and Bolshakov, 2019). Finally, somatosensory inputs received by the BLA are accurately processed within intra-amygdala micro-circuits and transmitted to the central nucleus to further mediate physiological manifestations of fear through projections to the hypothalamus and downstream structures in the brainstem (Keifer et al., 2015; Tovote et al., 2015; Carli and Farabollini, 2022) (Figure 13).

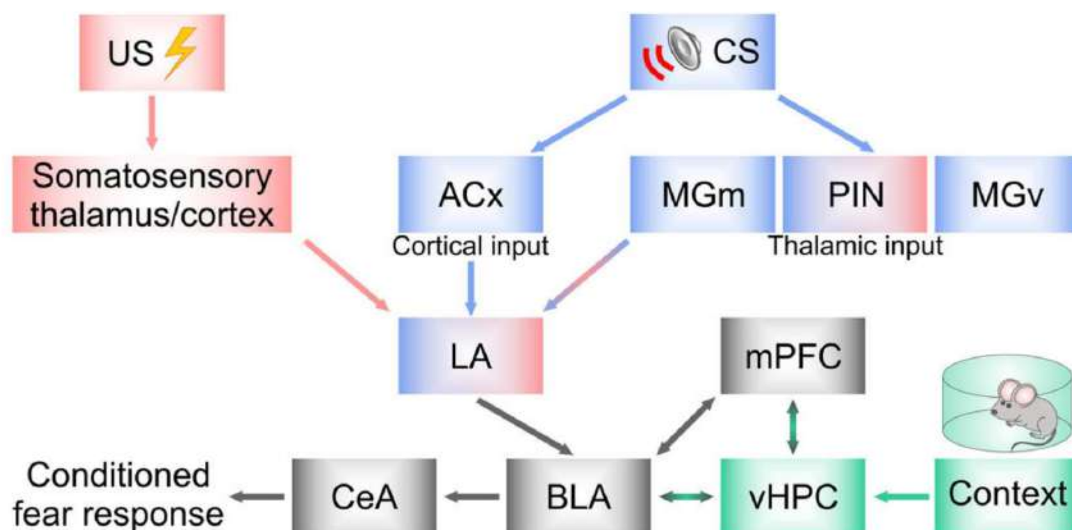

Figure 13. Diagram illustrating brain circuitry of fear-related learning. ACx, auditory cortex; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; CS, conditioned stimulus; LA, lateral nucleus of the amygdala; MGm, medial geniculate nucleus; MGv, ventral geniculate nucleus; mPFC, medial prefrontal cortex; PIN, posterior intralaminar nucleus of the thalamus; US, unconditioned stimulus; vHPC, ventral hippocampus (Adapted from Luchkina and Bolshakov, 2019).

Compared to fear learning or conditioning, fear extinction is the reverse process since it supresses the original aversive memory. The ability to correctly extinguish conditioned fear memories is essential for an adaptative control of fear response. Indeed, individuals with the aforementioned fear-related disorders show clear alterations on the underlying mechanisms regulating fear extinction, thus becoming a critical hallmark of such emotional disorders. The neural circuitry recruited for the extinction of fear memories is similar to that involved in fear learning or conditioning in both humans and rodents (Namkung et al., 2022). Accordingly, the amygdala presents a central role in this process by regulating the transition between states of high and low fear through two different populations of neurons in the basal nucleus. This region can rapidly switch the balance of activity between these opposite neuronal populations, depending on the afferent projections from the mPFC and the hippocampus (Herry et al., 2008). Interestingly, the mPFC is characterized by a profoundly studied ambivalence in the regulation of fear. Whereas PL is responsible for fear acquisition and expression, IL (ventromedial prefrontal cortex in humans) manages the extinction of aversive memories by suppressing signal flows within the amygdala and thereby decreasing fear response (Milad and Quirk, 2002; Milad et al., 2004; Bukalo et al., 2015). A key study performed by Sierra-Mercado and colleagues demonstrated that inactivation of IL with the $GABA_A$ receptor agonist muscimol, while preserving PL, impaired fear extinction in the auditory fear conditioning and extinction test, thus supporting the essential role of IL in the extinction of aversive memories (Sierra-Mercado et al., 2011). However, recent controversial research has pointed an indirect effect of PL in the extinction process via IL activation (Marek et al., 2018b). Analogous to fear conditioning, the hippocampus presents an important contribution to the extinction of fear memories, mainly those involving contextual cues (Sierra-Mercado et al., 2011; Lacagnina et al., 2019). During contextual fear extinction, the hippocampus prevents fear expression inhibiting output neurons in the central amygdala by direct activation of selected inhibitory neurons of the BLA (Farinelli et al., 2006), and indirectly activating IL neurons that projects to the BLA (Hugues et al., 2006). As a result, the central amygdala sends the final response to downstream structures in the brainstem, based on the afferent projections from the mPFC and the hippocampus. Moreover, inhibitory micro-circuits in this output region also act as a critical factor within the brain networks mediating fear extinction (Whittle et al., 2021).

3.2.2. Neurochemical substrates of fear learning and memory

The multiple underlying mechanisms modulating fear response are specific to each structural level. Therefore, several neurotransmitter and neuromodulatory systems

regulate neural projections carrying the CS and US to the BLA, synaptic plasticity modulators allow the association between both stimuli, and outputs from the central amygdala activate peripheral mechanisms, mainly the glucocorticoid system. This machinery accurately orchestrates the correspondent fear behaviour.

In the last few years, significant progress has been made to elucidate the global set of molecular systems modulating conditioned fear acquisition and extinction, in animal models. Interestingly, the endocannabinoid system plays an important role in stress recovery by regulating fear (Maldonado et al., 2020), thus becoming one of the main objectives of the current thesis. Hence, CB1R KO mice present clear deficiencies in short- and long-term extinction in auditory fear-conditioning tests, as well as rimonabant-treated wild-type mice. This behaviour remains unaltered in the acquisition of fear memory (Marsicano et al., 2002). Conversely, fewer and non-conclusive studies have investigated the role of CB2R in fear extinction. In this sense, a study performed with CB2R KO mice presented no differences in fear learning or conditioning, compared to wild-type animals. In accordance with these results, CB2R blockade with AM630 had no effect in the same behavioural test (Li and Kim, 2016). However, the role of CB2R in the extinction of fear memories has not been studied yet. This gap in knowledge represents one of the main objectives widely addressed along the present thesis (Articles 2 and 3). Besides the endocannabinoid system, other neurotransmitter systems are known to modulate fear response, including dopamine, noradrenaline, serotonin, GABA and glutamate (Bukalo et al., 2014). Analogously, diverse neuropeptides have also been shown to exert an important role in fear behaviour. As previously detailed, the orexin system is deeply involved in both fear consolidation and extinction (Flores et al., 2014). Elucidating the underlying mechanisms regulating such response is one of the main goals of this thesis (Article 2). Furthermore, other neuropeptides regulate fear response, such as neuropeptide Y and S, which administration enhances the extinction of fear memories (Gutman et al., 2008; Jüngling et al., 2008). Altogether, final balance from the multiple mediators involved in the transmission of fear-related stimuli is decisive for the subsequent fear response.

In order to achieve a suitable association between CS and US, and create fear memories, plastic changes among BLA neurons are required. To execute such remodelling process, the brain-derived neurotrophic factor (BDNF) is one of the main neurotrophins that promotes neuronal proliferation, synaptic plasticity, and long-term potentiation in the CNS (Kowiański et al., 2018). Hence, BDNF is described as an essential factor to properly encode fear memory acquisition and extinction, by interacting with tropomyosin-related kinase B (TrkB) receptor (Ou and Gean, 2006; Meis et al.,

2020). In accordance with this theory, heterozygous BDNF mice exhibited impaired fear learning (Meis et al., 2018), as well as conditional knockout BDNF mice presented deficiencies in the extinction of fear memories (Heldt et al., 2007). On the other hand, intra-IL administration of recombinant BDNF facilitates cued-fear extinction in rats (Peters et al., 2010). Other neurotrophic factors have also been associated to fear response, such as the tropomyosin-related kinase C (TrkC). Consistently, mice overexpressing TrkC receptor presented a strong fear-related phenotype, with increased anxiety-like behaviour and panic reaction (Dierssen et al., 2006). In conclusion, key elements modulating neural plasticity in fear-related structures, mainly in the BLA, are required for an appropriate fear learning and extinction.

Finally, to correctly generate fear response, efferent neurons from the central amygdala reach diverse downstream structures, including the hypothalamus, which initiates the HPA axis activation (Gray et al., 1989; Keifer et al., 2015). Briefly, HPA axis is commonly activated by stress-related stimuli, such as fear (Dunlop and Wong, 2019). Such neuroendocrine cascade begins with the release of CRF from the paraventricular nucleus of the hypothalamus. This hormone stimulates the secretion of ACTH in the anterior pituitary (also named adenohypophysis), that reaches the adrenal cortex and increases the production and release of cortisol in humans, or corticosterone in rodents (Spencer and Deak, 2017; Leistner and Menke, 2020). Among other functions, this steroid hormone facilitates active coping with threatening situations by binding to glucocorticoid and mineralocorticoid receptors, thus exerting both rapid, non-genomic and slow, genomic actions (de Kloet et al., 2008; de Quervain et al., 2017). It is also reported that glucocorticoid system enhances the consolidation and extinction of fear memories, since blockade of such receptors impair both behavioural processes, whereas agonism produces the opposite effect (Yang et al., 2006; Rodrigues et al., 2009; Blundell et al., 2011). For that reason, PTSD patients ameliorated fear-related symptoms with low doses of daily-administered cortisol (Aerni et al., 2004). Overall, peripheral systems are crucial elements for the execution of fear response, as well as for the regulation of fear learning and memories.

3.3. Research on fear dysregulations

Much of our understanding about the pathophysiology and the subsequent discovery of molecular targets for fear-related disorders is based on basic research. Hence, selecting the appropriate behavioural protocol to correctly assess fear response, and the most representative animal model that mimics the human disease, is crucial to increase our knowledge about such complex psychiatric disorders.

3.3.1. Experimental approaches of fear evaluation

Diverse behavioural tasks are commonly used in preclinical research to evaluate fear response in different conditions. Most of the tests are based on classical (Pavlovian) conditioning, which entails an associative learning through the relation between a neutral CS and an aversive US (Pavlov, 1927; Estes and Skinner, 1941). Once this association is encoded, re-exposure to the CS alone induces a fear response that can be quantified in a variety of ways, the most common are the freezing behaviour and the startle response, measured in the fear conditioning and the fear-potentiated startle tests, respectively (Figure 14).

The fear conditioning test has become part of the standard arsenal of behavioural tasks employed to measure fear response in animal models. First, animals are freely moving in a specific context to explore it and get habituated, until a neutral stimulus, such as a tone, light or the physical context surrounding the animal (CS), is repeatedly paired with an aversive stimulus, like an electric footshock (US). In order to achieve a fear memory consolidation, animals are usually housed in their cages for 24 hours. Then, they are placed in the same context or a different one with the CS alone. Given the CS-US association, subsequent re-exposure to CS induces a fear response that is measured by the freezing behaviour throughout the test. Since US is not presented anymore, animals start to dissociate both stimuli, thus decreasing the time animals spend freezing (Bouton and Bolles, 1980; Maren, 2001; Tovote et al., 2015). Conceptually, many authors describe this process as a new memory creation that is put before the fear memory (Myers and Davis, 2007; Quirk et al., 2010; Zhang et al., 2020). However, this aversive memory is not fully erased, as later exposure to CS still evokes a fear response, usually referred to as fear relapse (Yoshii et al., 2017; Lacagnina et al., 2019) (Figure 14A). Based on the same Pavlovian paradigm, fear-potentiated startle test is measured with the startle response amplitude in the presence of CS, compared to basal conditions (no CS) (Davis et al., 1993; Falls, 2002; Groenink et al., 2023). In this test, animals significantly increase their startle response under fear conditions and gradually decrease with repeated presentations of CS alone (Jones et al., 2005) (Figure 14B). Moreover, some other parameters can be recorded to physiologically determine the fear state during or after each test, such as heart rate and blood pressure (Davern and Head, 2011; Hsu et al., 2012; Liu et al., 2013).

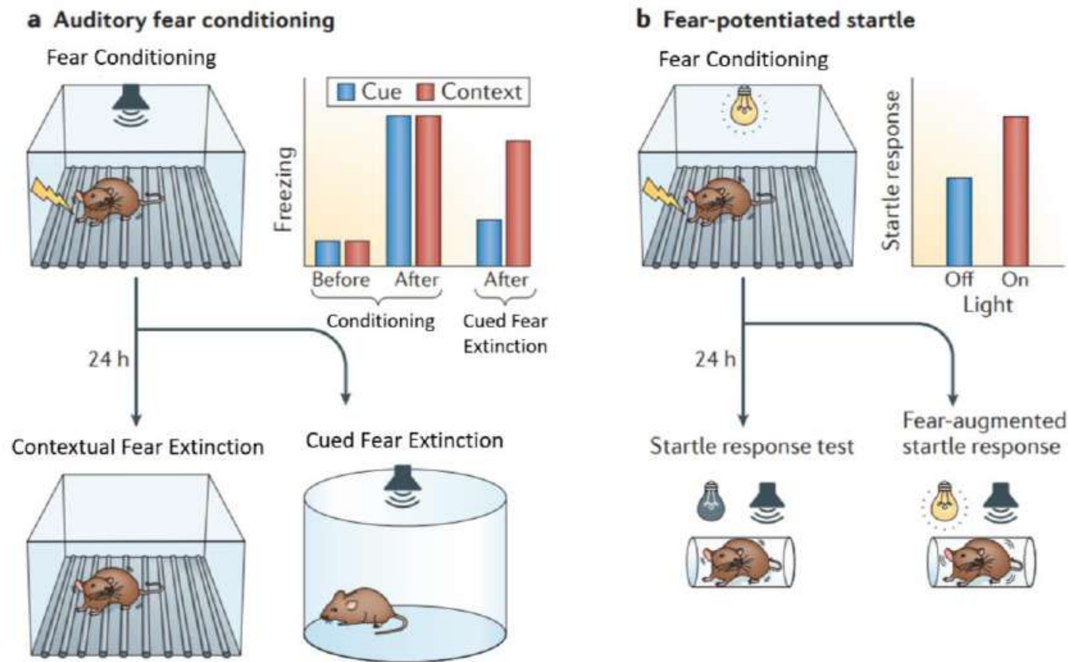

Figure 14. Behavioural tasks measuring fear response in rodent models. (A) Pavlovian fear conditioning and extinction and (B) fear-potentiated startle test (Modified from Tovote *et al*, 2015).

In contrast to the Pavlovian fear paradigm, operant conditioning (also named instrumental conditioning) can be used to assess another fear-related feature: threat avoidance. This test entails a behavioural pattern that is guided by the association of the stimuli with the subsequent punishment or reinforcement. Depending on the behavioural task, operant conditioning paradigm might entail the execution or suppression of the escape response during CS presentation in order to avoid deleterious consequences, in the active and passive avoidance test, respectively (Bolles, 1970; Jiao et al., 2015; LeDoux et al., 2017).

In a less invasive manner, similar behavioural tests assessing fear response have been employed in humans. Indeed, we can naturally undergo classical fear conditioning in the presence of traumatic events. For example, in 2017 a terrorist attack took place in the centre of Barcelona, when an uncontrolled-driven van crashed into pedestrians for more than 500 meters. Some witnesses and injured people were reported to develop some kind of fear-related disorders, since an aversive stimulus (i.e., the terrorist attack) was automatically associated with other neutral stimuli (e.g., the street where it happened) (Querol et al., 2021). Such events can be translationally reflected in experimental research with humans, by using the classical fear conditioning paradigm and recording

different outcomes, such as fear-potentiated startle eye blink response, heart period, pupil size and skin conductance response (Staib et al., 2015; Khemka et al., 2017; Bach et al., 2018; Bach and Melinscak, 2020). Creating a new fear memory to dissociate both stimuli is essential to correctly extinguish aversive memories, as demonstrated by preclinical research. As a consequence, extinction-based exposure therapies are commonly used for the treatment of fear-related disorders in order to alleviate anxiogenic symptomatology (Gonçalves et al., 2012; McLean et al., 2022).

3.3.2. Animal models of aberrant fear response

There is a pressing need to develop translationally-relevant animal models of impaired fear response, given the alarming epidemiology of anxiety disorders, which one of the main features is an excessive and enduring fear (Craske et al., 2017). The main impaired fear-related trait underlying these psychiatric diseases is the extinction of fear memories. Hence, preclinical research has focused on the accurate generation of animal models (mainly rodents) with alterations in such features, by disrupting a neural circuit or region, exposure to environmental insults, genetic manipulations, or the combination of different alterations (Goode and Maren, 2014; Singewald and Holmes, 2019). These experimental approaches arguably have a considerable predictive validity to elucidate important aspects of the pathophysiology of fear-related disorders.

Manipulating specific brain areas during fear behaviour evaluation is a useful tool to preclinically decipher the role of each region in fear extinction. The key brain areas involved in the extinction of aversive memories (i.e., amygdala, mPFC and hippocampus) have been widely studied by local lesioning, pharmacological activation/inactivation, electrical stimulation, interregional disconnection, and optogenetic approaches. Moreover, other secondary fear-related areas have been also analysed, such as the PAG, VTA, BNST, and striatum, among others (Tovote et al., 2015). An example of such experimental model is the study performed by Do-Monte and co-workers with male rats, in which optogenetic activation or silencing of glutamatergic neurons in IL during fear extinction and relapse allowed to better understand the role of IL in each fear process, in a neuronal- and temporal-specific manner (Do-Monte et al., 2015). Similar studies with this type of preclinical approach have revealed relevant results that increase our knowledge in this field (Sierra-Mercado et al., 2011; Arico et al., 2017; Bloodgood et al., 2018; Marek et al., 2018a). Thus, animal models with functional alterations in one of the multiple nodes of the neural fear circuitry report meaningful information about the role of such region in the fear response.

Animals models of impaired fear extinction can be also generated by exposure to environmental insults, such as stress, drugs and unhealthy diets, among others. These models represent a valuable translational approach since detrimental epigenetic factors are commonly related to our current society. In particular, stress has a leading role as a risk factor for the onset of anxiety, and trauma- and stressor-related disorders (American Psychiatric Association, 2013). As reported in one of the specific objectives of the present thesis, an extensive literature has described the effects of stress exposure in fear extinction in a wide variety of protocols, thus highlighting the importance of the type and chronicity of the stressor employed (Miracle et al., 2006; Maroun et al., 2013; Sillivan et al., 2017; Knox et al., 2018). In this sense, immobilization stress and single prolonged stress seem to induce a strong impairment on the extinction of aversive memories (Deslauriers et al., 2018). However, some other factors are also relevant for the effects of stress on fear extinction, including sex, age, and prior experience (Maren and Holmes, 2016). Concerning sex differences, an interesting study with male and female rats showed significant deficits in the extinction of fear memories in male, but not female rats, as a consequence of peri-pubertal stress exposure (Toledo-Rodriguez et al., 2012). Also, a growing body of evidence emphasizes late childhood/adolescence as a window of vulnerability for the impairment of fear extinction, thus underscoring life stage as a key element in these protocols (Baker et al., 2016). Aside from stress, consumption of drugs and unhealthy food triggers adverse effects on fear extinction, with special attention to the aforementioned factors in stress protocols. For example, alcohol intake during adolescence disrupted fear extinction by altering neural plasticity in mPFC, in both male and female mice (Lawson et al., 2022). A similar study with high-fat/high-sugar diet during adolescence revealed fear extinction deficits and anxiety-like behaviour during adulthood, in male rats. This group of animals also exhibited biochemical disturbances in IL, with fewer parvalbumin-expressing cells, increased levels of FosB, and a clear trend towards increased microglial activity (Baker and Reichelt, 2016). Overall, a wide range of environmental insults, applied singly or in combination, has been found to impair fear extinction in animal models, to further identify novel mechanisms and therapeutically normalize extinction.

Despite the moderate evidence considering genetics as a risk factor of developing a psychiatric disorder after trauma exposure (Stein et al., 2002; van Houtem et al., 2013; Purves et al., 2021), preclinical research has already generated diverse genetic models of impaired fear extinction. However, these models still remain in early stages of development and testing. Such experimental approaches are based on specific gene deletion or addition (e.g., knockout, knockin, overexpression), or a novel genetic

background (e.g., different animal strains). Hence, genetic engineering can alter the DNA makeup of an organism to obtain functional information of such gene-coded protein. In this vein, diverse genetically-modified animal models with disrupted fear extinction have been obtained by deleting, for instance, the serotonin transporter (5-HTT) gene, or by mutating the BDNF gene with a human polymorphism, among many other examples (Wellman et al., 2007; Soliman et al., 2010). On the other hand, fewer models are based on concrete mouse or rat strains presenting deficits in the extinction of fear memories, in order to identify potential genes involved in this function (Hefner et al., 2008; Mcguire et al., 2013). An appropriate example is the inbred mouse strain 129S1/SvImJ (S1), which characterization constitutes one of the main objectives of this thesis (Article 3). S1 mice show a clear impairment to extinguish both cued- and context-fear memories, in comparison to control mouse strains, such as C57BL/6J (BL6) (Hefner et al., 2008; Camp et al., 2012; Keum et al., 2016). Interestingly, S1 mice present similarities to fear-related patients, such as lower heart rate variability during fear extinction, and slow recovery of this parameter after fear relapse (Arditi-Babchuk et al., 2009; Camp et al., 2012; Chalmers et al., 2014). Neural activity in key fear-related areas is consistent with the phenotype observed, since an hyperexcitability has been detected in PL and central amygdala, and hypoactivity in IL and basal amygdala (Hefner et al., 2008; Fitzgerald et al., 2014; Park and Chung, 2019). Moreover, PL-BLA projections in S1 remained abnormally high after extinction compared to BL6 mice, whereas IL-BLA circuit failed to switch to an inhibitory pathway at the same time (Park and Chung, 2020).These observations have been also reported in PTSD patients during fear relapse, thus increasing the translational relevance of these studies (Milad et al., 2009; Garfinkel et al., 2014). Altogether, genetic animal models with disrupted fear extinction have made a significant contribution to the identification of novel genes and the corresponding proteins involved in the pathophysiology of fear-related disorders.

OBJECTIVES

General Objective

Increasing prevalence of psychiatric disorders characterized by the presence of pathological fear is reported in today's society. Therefore, the main goals of the present thesis are to identify potential risk factors that may induce the onset of these pathologies by focusing on THC and stress exposure, and to better understand the neurobiological mechanisms involved in fear dysregulations through the orexin system and a specific mouse model of aberrant fear extinction.

Specific Objectives

1. To evaluate the consequences of THC treatment, as well as the effects of concomitant THC and stress exposure during adolescence in the extinction of aversive memories in the adulthood (Article 1).
2. To analyse the neurobiological alterations of adolescent THC treatment, as well as THC and stress exposure, in the adulthood (Article 1).
3. To study the role of the endocannabinoid system in the impairment of fear extinction induced by OXA (Article 2).
4. To decipher the precise components of the endocannabinoid system and their neuroanatomical localization involved in the impairment of aversive memories promoted by OXA (Article 2).
5. To assess specific behavioural traits of the 129S1/SvImJ inbred mouse strain, a well-established mouse model of impaired fear extinction (Article 3).
6. To investigate the role of the endocannabinoid system in the observed phenotype of the 129S1/SvImJ inbred mouse strain (Article 3).

RESULTS

Article 1

Concomitant THC and stress adolescent exposure induces impaired fear extinction and related neurobiological changes in adulthood

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MAIN CONCLUSIONS OF THE ARTICLE

In this article, we observed an impaired fear extinction in adult male mice, as a consequence of the interaction between cannabis administration and stress exposure during the adolescence. This long-term behavioural impairment was associated with biochemical disturbances, including an altered glucocorticoid tone, dysregulations of the fear circuit, and structural plasticity alterations in the BLA.

My contributions in the present article are the participation in all the behavioural and biochemical experiments, excluding the radioimmunoassay experiment used to analyse plasma corticosterone levels and the immunoblot analysis of CB1R. I was also involved in data curation and the dissemination of results.

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Concomitant THC and stress adolescent exposure induces impaired fear extinction and related neurobiological changes in adulthood

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HIGHLIGHTS

- Concomitant THC and stress adolescent exposure impairs fear extinction in adulthood.
- Concomitant THC and stress adult exposure does not alter fear extinction.
- IL and BLA activity is reduced due to simultaneous THC/stress adolescent exposure.
- Concomitant THC/stress adolescent exposure alters BLA structural plasticity.

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Amygdala

ABSTRACT

Δ9-tetrahydrocannabinol (THC) consumption during adolescence is reported to be a risk factor for the appearance of psychiatric disorders later in life. The interaction between genetic or environmental events and cannabinoid exposure in the adolescent period can also contribute to exacerbate behavioural deficits in adulthood. Here we investigate the effects of THC treatment as well as the consequences of concomitant THC and stress exposure during adolescence in the extinction of fear memory in adult mice. Adolescent mice treated with THC and exposed to stress exhibit impaired cued fear extinction in adulthood. However, no effect was observed in animals exposed to these two factors separately. Notably, resistance to fear extinction was associated with decreased neuronal activity in the basolateral amygdala (BLA) and the infralimbic prefrontal cortex, suggesting a long-term dysregulation of the fear circuit. These changes in neuronal activation were paralleled with structural plasticity alterations. Indeed, an increase of immature dendritic spines in pyramidal neurons of the BLA was revealed in mice simultaneously exposed to THC and stress. Corticosterone levels were also enhanced after the cued fear conditioning session in the same experimental group. These results show that an interaction between cannabis exposure and stress during adolescence may lead to long-term anxiety disorders characterized by the presence of pathological fear.

1. Introduction

Cannabis remains the most widely used illicit substance worldwide. The regular use of cannabis often begins during adolescence which is of particular concern because this period is crucial to generate efficient neuronal pathways by constant neuroplastic shaping, synaptic reorganization and neurochemical changes (Sturman and Moghaddam, 2011). Pharmacological and/or environmental factors affecting the endocannabinoid system during the adolescent period can lead to altered brain maturation considering the important role played by this

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system in the neurodevelopmental changes that occur during this time (Fernández-Ruiz et al., 2000; Harkany et al., 2007). As a consequence, preclinical and epidemiological data suggest that adolescent cannabinoid exposure may increase the risk for the appearance of psychiatric diseases in adult life (Malone et al., 2010; Higuera-Matas et al., 2015), including emotional dysregulation. Recent studies relate cannabis use in adolescence with amygdala hypersensitivity to signals of threat (Spechler et al., 2015), and with an increased likelihood of having posttraumatic stress disorder symptoms in adulthood (Lee et al., 2018). Other disturbances associated with adolescent cannabis use may include psychotic-like symptoms, cognitive deficits, and increased addiction vulnerability (Silins et al., 2014; Renard et al., 2016).

Most psychiatric disorders involve multiple ethiopathological factors that can interact across the lifespan and trigger disease onset (Caspi and Moffitt, 2006). The interaction between cannabis consumption and genetic or environmental factors during adolescence may have a crucial influence in the detrimental effects of this drug later in life (Rubino and Parolaro, 2016). Adult mice with genetic mutations in some genes involved in schizophrenia (O'Tuathaigh et al., 2012; Long et al., 2013; Ballinger et al., 2015) or exposed to stressful events early in life (Llorente-Berzal et al., 2011; Zamberletti et al., 2012; Klug and van den Buuse, 2012) showed altered behavioural responses due to Δ^9-tetrahydrocannabinol (THC) adolescent treatment. This interaction of cannabis exposure and genetic/environmental events produces protective or negative effects depending on the genetic profile, sex, and stress level (Rubino and Parolaro, 2016).

The most consistent consequences of adolescent cannabinoid exposure are related to long-term impairments in cognitive function. Thus, numerous studies have shown working memory deficits in animals exposed to different cannabinoid agonists during adolescence (Renard et al., 2016). The consequences in emotional memory have been less studied, although adolescent THC administration did not produce lasting effects in this behaviour (Ballinger et al., 2015; Rubino et al, 2009a, 2009b). However, whether adolescent cannabinoid exposure alone or in combination with environmental factors such as stress affects the extinction of fear memory in adult life remains to be elucidated. Cannabis consumption and stressful events are often associated (Ketcherside and Filbey, 2015). The effects in adulthood of cannabis consumption during adolescence may be exacerbated by stress exposure in this period of life. Moreover, stress and fear responses share common neural circuits, and the neuronal structures involved in fear acquisition and extinction are also highly sensitive to stress effects (Stockhorst and Antov, 2016).

Here, we reveal that concomitant THC and stress adolescent exposure induces long-term impairment in fear extinction. This effect is associated with reduced neuronal activity and structural plasticity changes in key limbic brain regions. These findings suggest that THC chronic consumption under stress conditions during adolescence may increase the risk for the appearance of anxiety disorders related to trauma exposure.

2. Materials and methods

2.1. Animals

Adolescent female and male, and adult male C57BL6/J mice (Charles River, France) were used in these experiments. Animals were housed 3–5 per cage in a room with controlled temperature (21 ± 1 °C), and humidity (55 ± 10%) and with a 12 h light/12 h dark cycle. All behavioural studies were conducted during the light period. Food and water were available *ad libitum*. Animal procedures were performed in accordance with the guidelines of the European Communities Council Directive 2010/63/EU and approved by the local ethical committee (CEEA-IMAS-UPF), and the statement of compliance with standards for use of laboratory animals by foreign institutions nr. 5388-01 approved by the National Institutes of Health. All behavioural data were obtained by experimental observers blinded to the experimental conditions.

2.2. Drugs

THC stored at 100 mg/ml in ethanol (THC-Pharm-GmbH, Germany) was diluted in 5% Tween-80 and physiological saline solution to achieve doses of 3, 6 and 12 mg/kg (5 ml/kg of body weight).

2.3. Experimental designs

2.3.1. Adolescent THC treatment

The long-term effects of THC administration in anxiety-like responses, locomotor activity, and fear conditioning expression and extinction were evaluated in adolescent male and female mice. Adolescence, which is a vulnerable period for the onset of neuropsychiatric disorders, covers the complete time span from childhood (shortly before puberty) to adulthood, including the pubertal period (Schneider, 2013). The timing of human adolescence is difficult to define (Schneider, 2008), and therefore the exact timing of this period in laboratory rodents represents a challenge in animal research (Schneider, 2013). Starting at PND 35, mice were subcutaneously administered with increasing doses of THC (PND 35–39: 3 mg/kg, PND 40–44: 6 mg/kg, and PND 45–49: 12 mg/kg) or vehicle during 15 days in order to counter the development of drug tolerance (Renard et al., 2017). Similar protocols using escalating doses of THC during adolescence have been previously used in numerous studies (Rubino et al., 2008; Llorente-Berzal et al., 2013; Cadoni et al., 2015). Moreover, comparable time frames for adolescent cannabinoid treatment in both male and female rodents have been previously reported (Biscaia et al., 2003; Rubino et al., 2008; Realini et al., 2011; Bortolato et al., 2014; Zamberletti et al., 2014). Male and female mice could be at different stages of the development at the onset and during the treatment schedule. However, the objective of our study was to evaluate in adult mice the possible effects of THC treatment during the adolescent period, a crucial life stage for the neuronal development. From PND 50 to 69, animals remained undisturbed. At PND 70, behavioural evaluation was carried out in the order described in Fig. 1A for male and in Fig. S1 for female mice. The interval of time between adolescent THC or vehicle treatment and adult behavioural experiments is similar to those used in previous reports (Quinn et al., 2008; Llorente-Berzal et al., 2013). On the other hand, long-term stress of injection effects during adolescence (Keeley et al., 2015; Simone et al, 2018a, 2018b) have been previously reported, which may reflect a greater vulnerability of adolescent animals to repeated physical stress exposure. However, control groups were injected with vehicle in our experimental protocols suggesting that the effects observed in mice treated with THC were due to this drug.

2.3.2. Concomitant THC and stress adolescent exposure

To assess the interaction between THC and mild stress, adolescent male mice treated with THC or vehicle, as previously described, were exposed to different stressors: forced swimming, tail suspension and restraint. Similar stressors, applied singly or in combination, either acutely or chronically, have been used in previous studies to investigate the role of stress in fear processing (Maren and Holmes, 2016). At the end of each 5-days vehicle or THC exposure, one stressor was applied as shown in Fig. 2*A*. Moreover, two additional stressors were applied at PND 60 (forced swimming) and 65 (tail suspension) (Fig. 2*A*). Behavioural evaluation was carried out at PND 70 in the order shown in Fig. 2*A*.

2.3.3. Concomitant THC and stress adult exposure

To evaluate whether the long-term effects of simultaneous THC and stress exposure were age dependent, a similar experimental design was performed in adult male mice (Fig. S2*A*). Starting at PND 56, mice were subcutaneously administered with increasing doses of THC (PND

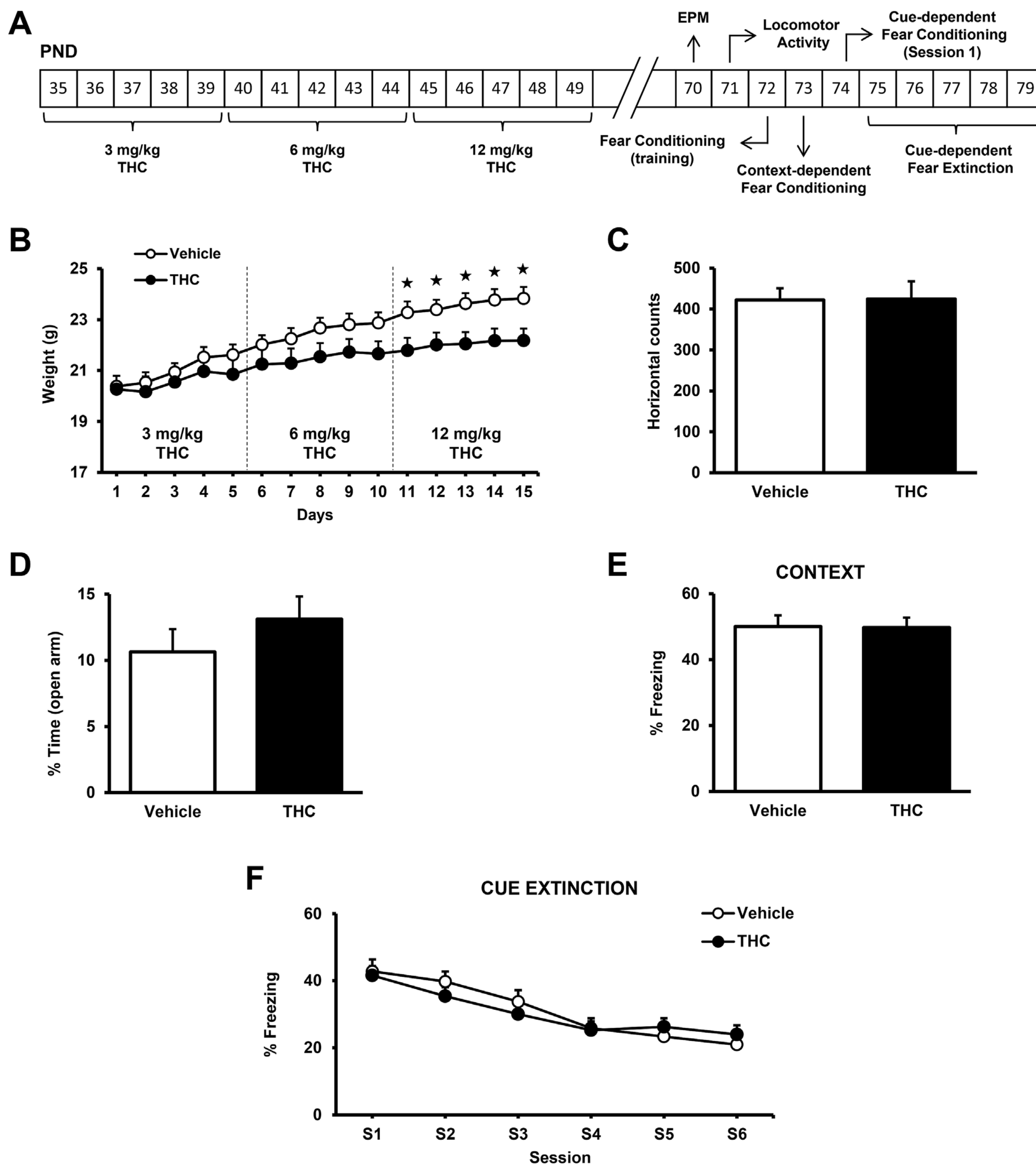

Fig. 1. Adolescent THC treatment does not modify fear memory processing in adult mice. (A) Schematic representation of the experimental design. Body weight of adolescent (B) male mice during the 15 days of treatment with THC (PND 35–39: 3 mg/kg, PND 40–44: 6 mg/kg, and PND 45–49: 12 mg/kg) or vehicle. Locomotor activity expressed as horizontal counts of adult (C) mice after adolescent THC or vehicle treatment. (D) Anxiety-like behaviour in the EPM expressed as the percentage of time spent in the open arm of adult mice after adolescent THC or vehicle treatment. (E) Freezing levels scored during contextual fear conditioning in adult mice treated with THC or vehicle during adolescence. (F) Freezing levels scored during cued fear conditioning (S1) in adult mice treated with THC or vehicle during adolescence. (F) Time course of the freezing levels scored during cued fear extinction trials in adult mice exposed to THC or vehicle during adolescence. Data are expressed as mean ± SEM (n = 10 mice per group). ★$p < 0.05$ (comparison between THC and vehicle). PND: postnatal day. EPM: elevated plus maze. S: session.

56–60: 3 mg/kg, PND 61–65: 6 mg/kg, and PND 66–70: 12 mg/kg) or vehicle during 15 days. At the end of each 5-days THC exposure one stressor was applied as previously described (Fig 2*A* and Fig. *S2A*). Two additional stressors were applied at PND 80 (forced swimming) and 85 (tail suspension). Behavioural evaluation was carried out at PND 90 in the order described in Fig. *S2A*.

2.4. Stress procedure

2.4.1. Forced swimming

Mice were placed in a clear Plexiglas cylinder containing water (20 ± 1 °C) for 6 min. The depth of the container and the volume of water were enough to prevent the animal from touching the bottom, thus forcing mice to swim.

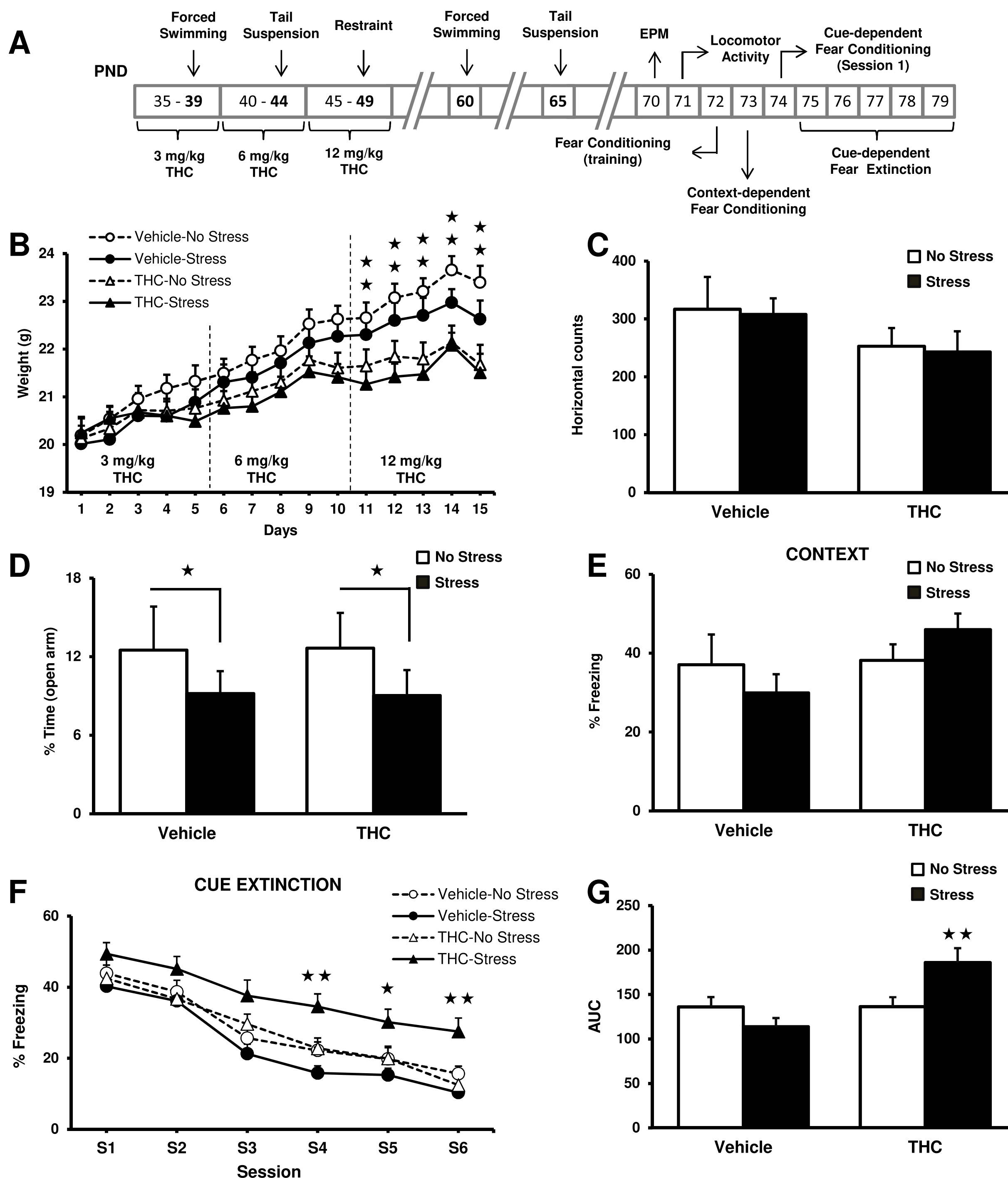

Fig. 2. Concomitant THC and stress exposure during adolescence impairs fear extinction in adulthood. (A) Schematic representation of the experimental design for (B–G). (B) Body weight of adolescent male mice during the 15 days of treatment with THC (PND 35–39: 3 mg/kg, PND 40–44: 6 mg/kg, and PND 45–49: 12 mg/kg) and stress exposure (n = 15–16 mice per group). (C) Locomotor activity expressed as horizontal counts and (D) anxiety-like behaviour in the EPM expressed as the percentage of time spent in the open arm in adult mice after adolescent THC, stress, or concomitant THC/stress exposure (n = 8 mice per group). (E,F) Freezing levels scored during (E) contextual and (F) cued fear conditioning (session 1) in adult mice after adolescent THC, stress, or concomitant THC/stress exposure (n = 15–16 mice per group). (F) Time course of the freezing levels scored during cued fear extinction trials in adult mice after adolescent THC, stress, or concomitant THC/stress exposure (n = 15–16 mice per group). (G) AUC values for the percentage of freezing during cued fear extinction trials. Data are expressed as mean ± SEM. ★$p < 0.05$; ★★$p < 0.01$ (comparison between THC and vehicle groups in (B); comparison between stress and non-stress mice in (D); comparison between THC/stress and THC/non-stress mice in (F) and (G)). PND: postnatal day. EPM: elevated plus maze. S: session. (Fisher LSD test).

2.4.2. Tail suspension

Animals were suspended by a 15 cm thin string hanged from a metal rod and stuck with adhesive tape 1 cm from the tip of the tail. The front paws were sufficiently distant to the ground to avoid mice touching it. Tail suspension was performed during 6 min.

2.4.3. Restraint

Stress was induced by immobilizing the animal with a restrainer apparatus. Mice were placed individually inside a 50 ml conical tube with 0.5 cm air holes for breathing without access to food or water for 30 min.

2.5. Behavioural experiments

2.5.1. Locomotor activity

Changes in horizontal activity were assessed by using locomotor activity boxes (9 × 20 × 11 cm, Imetronic, France). Mice were placed in locomotor cages with low luminosity. Activity was measured as the total number of horizontal photocell counts during 15 min.

2.5.2. Elevated plus maze

Elevated plus maze was performed to evaluate anxiety-like responses (Rubino et al., 2008). The maze consisted of four arms (16 × 5 cm) extended from a central square (5 × 5 cm) shaping a cross. Two opposite arms were delimited by vertical walls (closed arms), whereas the two other opposite arms had unprotected edges (open arms). The apparatus was elevated 30 cm above the floor and indirectly illuminated from the top (50–60 lux in the open arm). A 5 min trial was conducted by placing each animal in the central square and facing one of the open arms. The performance was recorded with a video camera system located above the maze. Results are expressed as the total entries to the closed and open arms, and the percentage of time spent in the open arms with respect to the total amount of time spent in both arms. An arm entry was counted when the animal moved both front paws into the arm.

2.5.3. Fear conditioning

Training and testing were conducted as described previously with some modifications (Bilkei-Gorzo et al., 2012; Na et al., 2012; Flores et al., 2014; Soria-Gómez et al., 2015). Mice were individually placed in a shuttle chamber (LE918, Panlab, Barcelona) surrounded by a sound-attenuating cabinet (Flores et al., 2014). The chamber floor was formed by parallel stainless-steel bars connected to a scrambled shock generator. On the training day, mice were habituated to the chamber during 180 s before the exposure to an acute beeping 30 s sound (80 dB) repeated 3 times with a 10 s silenced interval. Each animal received an unconditioned stimulus (US) (0.7 mA footshock during 2s) paired with the end of each sound (conditioned stimulus, CS). After the third shock, the animal remained 30 s in the shuttle chamber. To test context-induced fear conditioning, mice were placed in the shuttle chamber 24 h after the training. Fear memory was assessed as the percentage of time that mice spent freezing during the first 3 min. Freezing response, a rodent's natural response to fear, was evaluated by direct observation and defined as complete lack of movement, except for respiration for more than 1 s. To evaluate cued fear conditioning (session 1), mice were re-exposed to the CS in a novel environment (a wide dark cylinder) 24 h after context acquisition. Mice were allowed to adapt for 3 min to the new environment which was followed by 30 s of the sound used in the training day. This sound was repeated 4 times with a 10 s interval. Freezing was scored during the time the sound was active. After the last sound trial, mice remained in the cylinder for 30 s.

2.5.4. Extinction training

Extinction training was initiated 24 h after the cue-dependent fear conditioning test. Mice were placed in the fear conditioning cylinder with a novel environment as described above. Mice were given once daily extinction training sessions for 5 days (sessions 2–6). The percentage of freezing time was calculated by following the same experimental procedure as in the session 1. The habituation time was reduced to 1 min as mice were previously adapted to the new context. Data from fear extinction in adult male mice exposed to THC and stress during the adolescent period were expressed as percentage of freezing behaviour and as area under the curve (AUC). AUC was calculated by using a standard trapezoid method, $AUC = [0.5 \times (B1 + B2) \times h] + [0.5 \times (B2 + B3) \times h] + \ldots [0.5 \times (Bn + Bn_{+1}) \times h]$, where Bn were the percentage of freezing behaviour for each mouse and h was the time (days) passed between the consecutive measurements (Gibaldi and Perrier, 1975).

2.6. Immunoblot analysis

Amygdala tissue was extracted 25 days after the last THC or vehicle administration (PND74). Tissue was immediately frozen and store at −80 °C. Amygdala was homogenized in 30 vol lysis buffer containing protease and phosphatase inhibitors. Samples were then centrifugated to eliminate any solid residue. Equal amounts of protein samples (20μg/well) were separated in 10% polyacrylamide gels before electrophoretic transfer onto to nitrocellulose membranes (Bio-Rad). Membranes were blocked during 1 h in 5% bovine serum albumin (BSA)-T-TBS prior incubation for 2 h with the primary antibodies: CB1 cannabinoid receptor (CB1R) (rabbit polyclonal, 1:500) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, mouse monoclonal, 1:15000) form Frontier Institute (Cb1-Rb-Af380-1) and Santa Cruz Biotechnology (sc-32233), respectively. Then, membranes were rinsed 3 times and incubated for 1 h with their corresponding secondary antibodies coupled to horseradish peroxidase: rabbit (1:10000) and mouse (1:10000) from Cell Signaling. Immunochemiluminescence was produced by incubation of the membranes with West-femto ECL substrate (Thermo Fisher Scientific). Images of immunoreactive bands were acquire on a ChemiDoc XRS System (Bio-Rad) and quantified by The Quantity One software v4.6.3 (Bio-Rad). The values obtained for CB1R were normalized to the detection of GAPDH in the same sample and expressed as a percentage of the control group (Vehicle-Non stress) (Fig. S3).

2.7. Plasma corticosterone quantification

Blood samples were collected at different time points: immediately after restraint (PND 49), at PND 67 (basal), and 30 min after training (PND 72), cued-fear conditioning (session 1) (PND 74) and cued-fear extinction (session 5) (PND 78) (Fig. 3*A*). Blood samples were obtained from the tail in tubes containing ethylenediaminetetraacetic acid. Double-antibody radioimmunoassay (RIA) was used to determine plasma corticosterone levels. RIA used 125I-corticosterone-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Spain), synthetic corticosterone (Sigma, Spain) as the standard, and an antibody raised in rabbits against corticosteronecarboxi-methyloxime-BSA kindly provided by Dr G. Makara (Institute of Experimental Medicine, Budapest, Hungary). Plasma corticosteroid-binding globulin was inactivated by low pH. All samples to be statistically compared were quantified in the same assay to avoid inter-assay variability.

2.8. Immunofluorescence

Two h after the last cued fear extinction session or just before behavioural testing, mice were deeply anesthetized by ip injection (0.2 ml/10g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intracardiac perfusion. Mice were intracardically perfused with 4% paraformaldehyde (PFA) solution. Brains were removed from skull and post-fixed in PFA for 24 h at 4 °C. Then, brains were transferred to a solution of 30% sucrose in PB 0.1M and kept at 4 °C. Coronal sections of 30 μm containing the prelimbic (PL) and infralimbic (IL) prefrontal cortex (from bregma 1.98 mm–1.54 mm) and the basolateral amygdala (BLA) (from bregma −1.22 mm to −1.82 mm) were obtained using a microtome. Brain slices were stored in a solution of 5% sucrose PB 0.1M. Free floating slices were rinsed in PB 0.1M and after blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 in PB 0.1M (DS-T-PB) during 2 h at room temperature. Slices were incubated overnight with the primary antibody anti-*c*-Fos in DS-T-PB at 4 °C (1:500, rabbit, Santa Cruz Biotechnology) (sc-7202). Next day, after three rinses with PB 0.1M, slices were incubated with the secondary antibody AlexaFluor-555 donkey anti-rabbit (1:500, Life Technologies) at room temperature for 2 h in DS-T-PB. Then, slices were rinsed three times and mounted with Mowiol onto glass slides coated with gelatin.

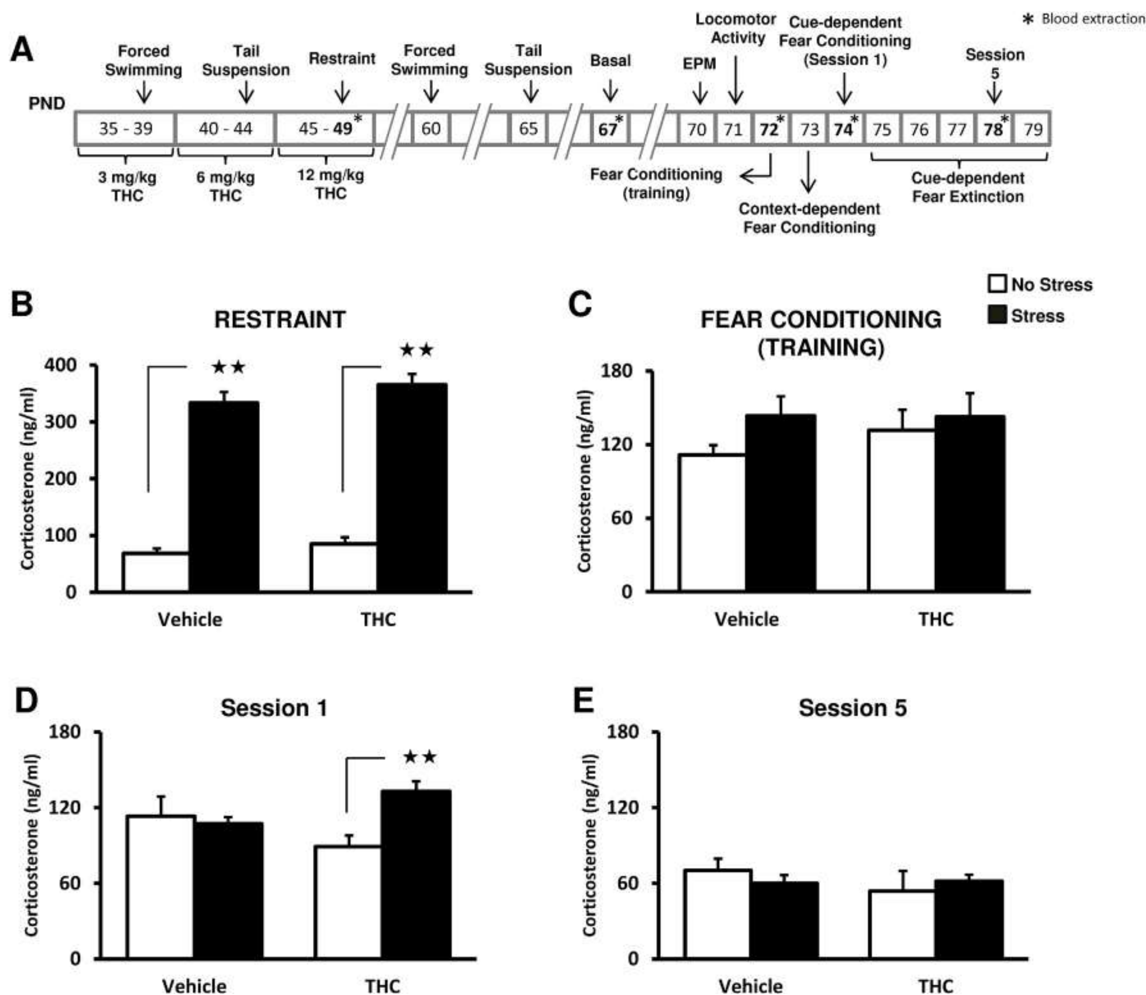

Fig. 3. Concomitant THC and stress exposure during adolescence increases corticosterone plasma levels after cued fear conditioning in adulthood. (A) Schematic representation of the experimental design for (B–E). Blood samples were collected at different time points, as indicated by the asterisk, and plasma corticosterone levels were measured (B) immediately after restraint (PND 49), 30 min after (C) fear conditioning training (PND 72), (D) cued fear conditioning (session 1) (PND 74) and (E) cued fear extinction (session 5) (PND 78). Data are expressed as mean ± SEM (n = 7–8 mice per group). ★★$p < 0.01$ (comparison between stress and non-stress mice in (B); comparison between THC/stress and THC/non-stress mice in (D)). PND: postnatal day. (Fisher LSD test).

2.9. Immunofluorescence image analysis

The stained sections were analyzed at 10× objective using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera Leica DFC 300FX (Leica Microsystems). For PL and IL analysis, a 430 μm sided square region of interest (ROI) was delimited for quantification. The expression of cFos in the images was quantified using the ImageJ analysis software. The option "particle counting" under a fixed threshold configuration was used to detect cFos positive cells. For all areas, 4 images per mice were quantified. Data are expressed as density of cFos positive cells per mm^2 (6–9 mice per each experimental condition).

2.10. Ballistic labeling with the fluorescent dye DiI

Mice were deeply anesthetized after the last cued fear extinction session by ip injection (0.2 ml/10g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intracardiac perfusion. Mice were perfused with 10 ml of PBS 0.1M, pH 7.5 followed by 40 ml of 4% (PFA) in PB 0.1M. Brains were postfixed in 4% PFA for 10 min. Then, brains were kept in PBS 0.1M for 12 h. Brain coronal sections (100 μm) containing the amygdala (from bregma −1.22 mm to −1.82 mm) were obtained by using a vibratome (Leica VT 1000 S) and kept in PBS 0.1M until fluorescent labeling processing. Brain slices were labeled by ballistic delivery of fluorescent dye DiI (Molecular Probes) using a gene gun apparatus (Helios Gene Gun System, Bio-Rad) as described previously (Gan et al., 2009) and postfixed with PFA for 4 h at room temperature to further preserve structures and to allow the diffusion of the dye DiI. Sections were placed on microscope gelatine-coated slides and coverslipped with mounting medium (Mowiol). Images were acquired using a confocal microscope (Leica TCS Sp5 STED) with a glycerol immersion lens (63X/1.30). Individual pyramidal neurons from the BLA were chosen for spine analysis based on several criteria, as previously described (Saravia et al., 2017). Briefly, (i) there was minimal or no overlap with other labeled cells to ensure that processes from different cells would not be confused, (ii) at least 3 primary dendrites needed to be visible for cells to be used for analysis and (iii) distal dendrites (from secondary dendrites to terminal dendrites) were examined. In addition, we chose only one apical or basal dendrite per neuron and quantified a minimum of 6 dendrites per animal. To calculate spine density, a minimum dendrite length of 20 μm long was required. All images of dendrites were taken at different z levels (0.13 μm depth intervals) to examine the morphology of dendritic spines. Reconstruction of dendrites and spine classification was performed using the IMARIS software (Bitplane). Protrusions from dendrites were classified into 5 types based on their morphology: class 1 or stubby protuberances were 0.5 μm in length, lacked a large spine head, and did not appear to have a neck; class 2, or mushroom-shaped spines

were between 0.5 and 1.25 µm in length and were characterized by a short neck and large spine head; class 3, or thin spines ranged between 1.25 and 3.0 µm and had elongated spine necks with small heads; class 4, or filipodia were 1.0 and 2.5 µm in length and were characterized by a large neck without spine head; and class 5 or branched spine ranged between 1.25 and 3.0 µm and had elongated spine necks with 2 or more spine heads. Quantification of dendritic spine densities was performed in blind conditions.

2.11. Data analysis

Data were analyzed by using unpaired Student t-test, two-way ANOVA or two-way ANOVA with repeated measures followed by subsequent post hoc analysis (Fisher LSD) when required. The Pearson correlation coefficient was used to analyse the relationship between cFos expression and freezing values. A p value < 0.05 was used to determine statistical significance. The statistical analysis was performed using STATISTICA (StatSoft) software.

3. Results

3.1. Adolescent THC treatment does not modify fear extinction in adulthood

Adolescent male mice were treated with increasing doses of THC during 15 days (PND 35–39: 3 mg/kg, PND 40–44: 6 mg/kg, and PND 45–49: 12 mg/kg). Body weight was daily evaluated along THC treatment. When mice reached adulthood, the effects of adolescent THC administration on fear memory processing were analyzed (Fig. 1*A)*. Possible changes in locomotor activity and anxiety-like behaviour were also examined (Fig. 1*A*). The weight gain of mice treated with THC was lower than those exposed to vehicle (Fig. 1*B*), as previously reported (Rubino et al., 2008; Stopponi et al., 2014; Scherma et al., 2016). An anxiogenic-like effect induced by a high dose of THC could explain the changes in body weight. Indeed, one week after the finishing of the treatment, a higher level of anxiety-like behaviour in adolescent rodents exposed to THC compared to controls was found in the elevated plus maze (EPM) test in previous studies (Stopponi et al., 2014). Nonspecific inhibition of ingestion, secondary to the sedative effects of THC could also be involved in this effect on body weight. Locomotor activity ($p = 0.96$, Student *t*-test) (Fig. 1*C*) and anxiety-like responses ($p = 0.44$, Student *t*-test) (Fig. 1*D*) (Fig. S2*A*) of adult mice were not affected by the chronic treatment with THC. The administration of THC did not modify the acquisition of fear memory as shown by similar freezing behaviour in both contextual ($p = 0.94$, Student *t*-test) and cued ($p = 0.78$, Student *t*-test) fear conditioning tests (Fig. 1*E* and *F* (Session1)). In addition, cued fear extinction was similar in adult animals exposed to THC or vehicle (Fig. 1*F*). Thus, two-way ANOVA of repeated measures showed no interaction between THC treatment and day ($F_{5,130} = 1.22$, $p = 0.30$) (Fig. 1*F*).

In an additional experiment, fear memory processing was not modified either in female mice (Fig. S1), in spite of the different sensitivity observed in several behavioural responses after treatment with cannabinoid agonists between sexes (Rubino and Parolaro, 2011). Our results indicate that THC administration during adolescence does not alter locomotion, anxiety and fear memory processing in adult male and female mice.

3.2. Concomitant THC and stress exposure during adolescence impairs fear extinction in adulthood

Cannabis consumption and environmental factors, such as stressful events, often coexist during adolescence and these factors could exacerbate the detrimental effects of cannabis use in adulthood. To test this hypothesis, we evaluated the consequences of simultaneous adolescent THC and stress exposure on locomotion, anxiety-like behaviour and fear memory processing in adult male mice. For this purpose, one stressor was applied at the end of each THC exposure period as shown in Fig. 2*A*. Two additional stress exposures were applied before behavioural evaluation (Fig. 2A). Adolescent mice receiving THC, independently of stress exposure, showed again a significant resistance to gain weight (Fig. 2B). Locomotor activity during adulthood was not modified by THC treatment, stress exposure or by the interaction of both factors (Fig. 2*C*). Mice that underwent stress, independently of THC exposure, showed an increase in anxiety-like behaviour. Thus, adolescent stressed mice spent significantly less time in open arms in the EPM (stress effect: $F_{1,60} = 4.17$, $p < 0.05$) (Fig. 2*D*), without significant interaction between THC and stress. This effect was not due to differences in the total number of entries (Fig. S2*B*). The acquisition of fear memory was not altered in adult mice exposed to stress, THC or both factors simultaneously during adolescence. Thus, freezing levels were similar in both contextual ($F_{1,58} = 2.62$, NS) and cued ($F_{1,59} = 2.69$, NS) fear conditioning tests (Fig. 2*E* and *F* (Session 1)). Notably, concomitant THC and stress exposure impaired cued fear extinction (Fig. 2*F*). Two-way ANOVA with repeated measures revealed a significant interaction between THC and stress ($F_{1,59} = 9.38$, $p < 0.01$), without other two-way or three-way interactions. Two-way ANOVA analyzed for each individual session revealed a significant interaction between stress and THC from session 4 to session 6 ($F_{1,59} = 10.26$, $p < 0.01$ at session 4; $F_{1,59} = 5.45$, $p < 0.05$ at session 5; $F_{1,59} = 15.56$, $p < 0.01$ at session 6). Subsequent post hoc analysis showed higher freezing levels in mice simultaneously exposed to THC and stress in comparison with those treated with THC ($p < 0.05$ at session 5; $p < 0.01$ at session 4 and 6), exposed to stress ($p < 0.01$ at session 4, 5 and 6), and vehicle non-stress mice ($p < 0.01$ at session 4 and 6; $p < 0.05$ at session 5) (Fig. 2*F*). In agreement, freezing levels expressed as AUC (Fig. 2*G*) were higher in mice exposed simultaneously to THC and stress in comparison with the other experimental conditions, as revealed by two-way ANOVA (interaction THC x stress: $F_{1,59} = 8.51$, $p < 0.01$) and post hoc analysis ($p < 0.01$). We next evaluated whether the impairment in cued fear extinction could be related to an alteration of CB1R levels in the amygdala. CB1R in this brain region plays a crucial role in the modulation of fear processing (Gunduz-Cinar et al., 2013). Western blot analysis showed no differences in total CB1R levels in the amygdala of adult mice exposed to THC and/or stress during the adolescent period (interaction THC x stress: $F_{1,19} = 1.99$, $p = 0.17$) (Fig. S3). This result suggests that, under our experimental conditions, impairment in fear extinction is not influenced by a reduced expression of CB1R in the amygdala of adult animals. However, changes in the efficacy or efficiency of the receptor binding can occur independently from the status of the receptor density, contributing to the observed phenotype.

To elucidate whether immature brain represents a period of development more susceptible to the effects of THC and stress, we compared the consequences of exposing the same type of stressors and THC treatment during adolescence and adulthood period (Fig. S4*A*). For this purpose, changes in behaviour in adult male animals were evaluated exactly 20 days following the final day of THC administration (Fig. S4A), as previously studied after exposure during the adolescent period. Notably, no differences in cued fear processing were observed between the different experimental groups exposed to THC and/or stress in adulthood period (Fig. S4*B* and *C*), suggesting that adolescence is a sensitive window for the harmful consequences of concomitant THC and stress exposure.

3.3. Concomitant THC and stress exposure during adolescence increases corticosterone levels following cued fear conditioning session

Stress during early-stages can impair fear extinction by modulation of glucocorticoid activity (Green et al., 2011). Therefore, we next evaluated possible changes in plasma corticosterone levels due to THC and stress exposure during adolescence at different time points (Fig. 3*A*). Corticosterone levels measured immediately after restraint

stress exposure were significantly increased in stressed mice (stress effect: $F_{1,27} = 300.43$, $p < 0.01$) (Fig. 3*B*), without influence of THC treatment (Veh-non stress: 68.38 ± 8.8 ng/ml; Veh-stress: 333.57 ± 11.57 ng/ml; THC-non stress: 82.25 ± 19.44; THC-stress: 365.38 ± 19.12 ng/ml). Basal levels of corticosterone after previous THC and stress exposure (PND 67) were unaltered in the different experimental groups (Fig. S5). Corticosterone levels measured 30 min after fear conditioning training were higher than in basal conditions, but remained similar in all experimental groups (Fig. 3*C*). Interestingly, previous simultaneous exposure to THC and stress increased plasma corticosterone 30 min after cued fear conditioning testing, as shown by two-way ANOVA (interaction treatment x stress: $F_{1,27} = 5.76$, $p < 0.05$) (Fig. 3*D*), and post hoc analysis ($p < 0.01$), THC-stress (132.88 ± 8.06 ng/ml) versus THC-non stress mice (89.13 ± 5.33 ng/ml) (Fig. 3*D*). This imbalance of corticosterone activity was temporal since similar levels were again observed after the fifth cued extinction session (Fig. 3*E*). These results suggest that glucocorticoid sensitization after cued fear conditioning may contribute to the extinction deficits revealed in mice exposed to THC and stress during the adolescent period.

3.4. *Impaired fear extinction induced by concomitant THC and stress exposure is associated with reduced activity of the infralimbic prefrontal cortex and the basolateral amygdala*

To identify the brain areas responsible for the resistance to fear extinction induced by simultaneous THC and stress exposure, we analyzed the possible activation of brain regions closely involved in the extinction circuit by using cFos immunofluorescence. cFos expression in the PL, IL and BLA was evaluated 2 h after the last cued fear extinction session, as behavioural alteration remained present at this time point. cFos expression in the PL was not altered in the different experimental groups ($F_{1,20} = 1.05$, NS) (Fig. S6). Interestingly, IL activation was reduced by the previous concomitant exposure to THC and stress ($p < 0.01$), as shown by two-way ANOVA (interaction treatment x stress $F_{1,32} = 6.70$, $p < 0.05$) (Fig. 4*A* and *B*). Values of cFos expression in the IL for the different experimental groups were the following: Veh-non stress: 247.10 ± 16.10 cFos + cells/mm^2; Veh-stress: 249.47 ± 25.36 cFos + cells/mm^2; THC-non stress: 253.76 ± 8.92 cFos + cells/mm^2; THC-stress: 171.00 ± 12.16 cFos + cells/mm^2). Likewise, simultaneous THC and stress adolescent exposure reduced the activity of the BLA as revealed by two-way ANOVA (interaction treatment x stress $F_{1,28} = 4.51$, $p < 0.05$), and post hoc analysis ($p < 0.05$) (Fig. 4*C* and *D*). Values of cFos expression in the BLA for the different experimental groups were the following: Veh-non stress: 144.03 ± 12.21 cFos + cells/mm^2; Veh-stress: 149.97 ± 14.12 cFos + cells/mm^2; THC-non stress: 138.60 ± 7.98 cFos + cells/mm^2; THC-stress: 108.83 ± 9.75 cFos + cells/mm^2). These results show that the impaired fear extinction induced by adolescent THC and stress exposure is associated with reduced activity of the IL and the BLA, which are key regions involved in the extinction of aversive memories. Indeed, a significant negative correlation ($p < 0.05$) between fear memory (freezing values) and IL activity (density of cFos + cells) was observed (Fig. 4*E*), whereas a clear tendency ($p = 0.058$) was revealed in the BLA (Fig. 4*F*). In an additional experiment cFos expression was analyzed in the BLA and the IL in adult mice exposed to THC and stress during the adolescence just before and after the fear conditioning and extinction paradigm. These experiments were carried out in order to investigate whether the reduction in the activity of these brain areas was due to the THC-stress treatment or if these changes were influenced by the dynamic interplay among THC, stress and fear conditioning/extinction. Simultaneous THC and stress adolescent exposure reduced the activity of the BLA (interaction THC x stress: $F_{1,23} = 9.52$, $p < 0.05$) (Fig. S7*A*) and the IL (interaction THC x stress: $F_{1,22} = 5.66$, $p < 0.05$) (Fig. S7*B*) after cued fear extinction, as previously reported. Interestingly, cFos expression was higher in adult animals exposed to THC and stress during the adolescence that did not undergo fear conditioning/extinction in both the BLA ($p < 0.05$) (Fig. S7*A*) and the IL ($p < 0.01$) (Fig. S7*B*) in comparison with mice that followed the behavioural paradigm. These results suggest that the reduced activity observed in the BLA and the IL depends on the dynamic effect of THC-stress exposure followed by fear conditioning and extinction behavioural testing.

3.5. *Impaired fear extinction induced by concomitant THC and stress exposure is associated with structural plasticity alterations in the basolateral amygdala*

Stress has been reported to alter structural plasticity in the amygdala (Leuner and Shors, 2013), affect fear extinction (Maren and Holmes, 2016), and THC exposure during adolescence can induce changes in dendritic spines in several brain regions (Rubino and Parolaro, 2016). Given the crucial role played by the BLA in fear extinction, we investigated whether concomitant exposure to THC and stress could affect structural plasticity in this brain area. Mice were sacrificed after the last cued extinction session and brains were processed for ballistic delivery to label whole neurons with the dye DiI. Total dendritic spine density of BLA pyramidal neurons was not modified by THC, stress or by the combination of both factors (Fig. 5*A*). Dendritic spines are dynamic and can be classified into different categories depending on their morphology (stubby, mushroom, thin, branched and filopodia). Density of mushroom (mature) spines was reduced in adolescent mice treated with THC (independently of stress exposure) ($p < 0.01$), as revealed by two-way ANOVA (treatment effect: $F_{1,23} = 9.35$, $p < 0.01$) (Fig. 5*B* and *C*). THC treatment increased the density of thin (immature) spines (treatment effect: $F_{1,23} = 7.43$, $p < 0.05$) (Fig. 5*B* and *C*). Notably, this effect was mainly due to the concomitant exposure to THC and stress as revealed by post hoc comparisons. Thus, THC-stress mice showed higher density of thin spines in comparison with THC-non stress ($p < 0.05$), vehicle-stress ($p < 0.05$), and vehicle-non stress ($p < 0.01$) mice (Fig. 5*B* and *C*). These data suggest that changes in the density of immature spines in the BLA might be involved in the impairment of fear extinction associated with the combination of THC and stress exposure during the adolescence.

4. Discussion

To the best of our knowledge, this is the first study demonstrating synergistic detrimental effects of simultaneous adolescent THC and stress exposure on the extinction of aversive memory in adulthood. Notably, impaired fear extinction was associated with a temporal imbalance of plasmatic corticosterone levels, decreased activity of key regions involved in fear regulation, such as the IL and the BLA, and changes in structural plasticity revealed by increased immature dendritic spines density in pyramidal neurons of the BLA.

The administration of THC during adolescence in male and female mice did not induce any modification in locomotor activity, anxiety-like responses, and fear regulation later in life. Controversial results have been previously reported after adolescent THC exposure since general anxiety by using EPM was not modified in adult rats (Rubino et al., 2008; Cadoni et al., 2015), whereas an anxiogenic-like effect was revealed in adult CD1 mice (Murphy et al., 2017). In agreement with our data, no lasting effects by adolescent THC exposure were previously observed in aversive memory as revealed by the lack of deficits in passive avoidance task (Rubino et al, 2009a, 2009b) or cued and contextual fear conditioning (Ballinger et al., 2015). However, a long-lasting impairment of fear conditioning has been described after chronic administration of the synthetic cannabinoid WIN 55,212-2 during adolescence (Gleason et al., 2012; Tomas-Roig et al., 2017), which has a higher intrinsic activity on cannabinoid receptors than THC (Kuster et al., 1993). On the other hand, repeated adolescent CB1R antagonism induced greater contextual fear memory recall in adult

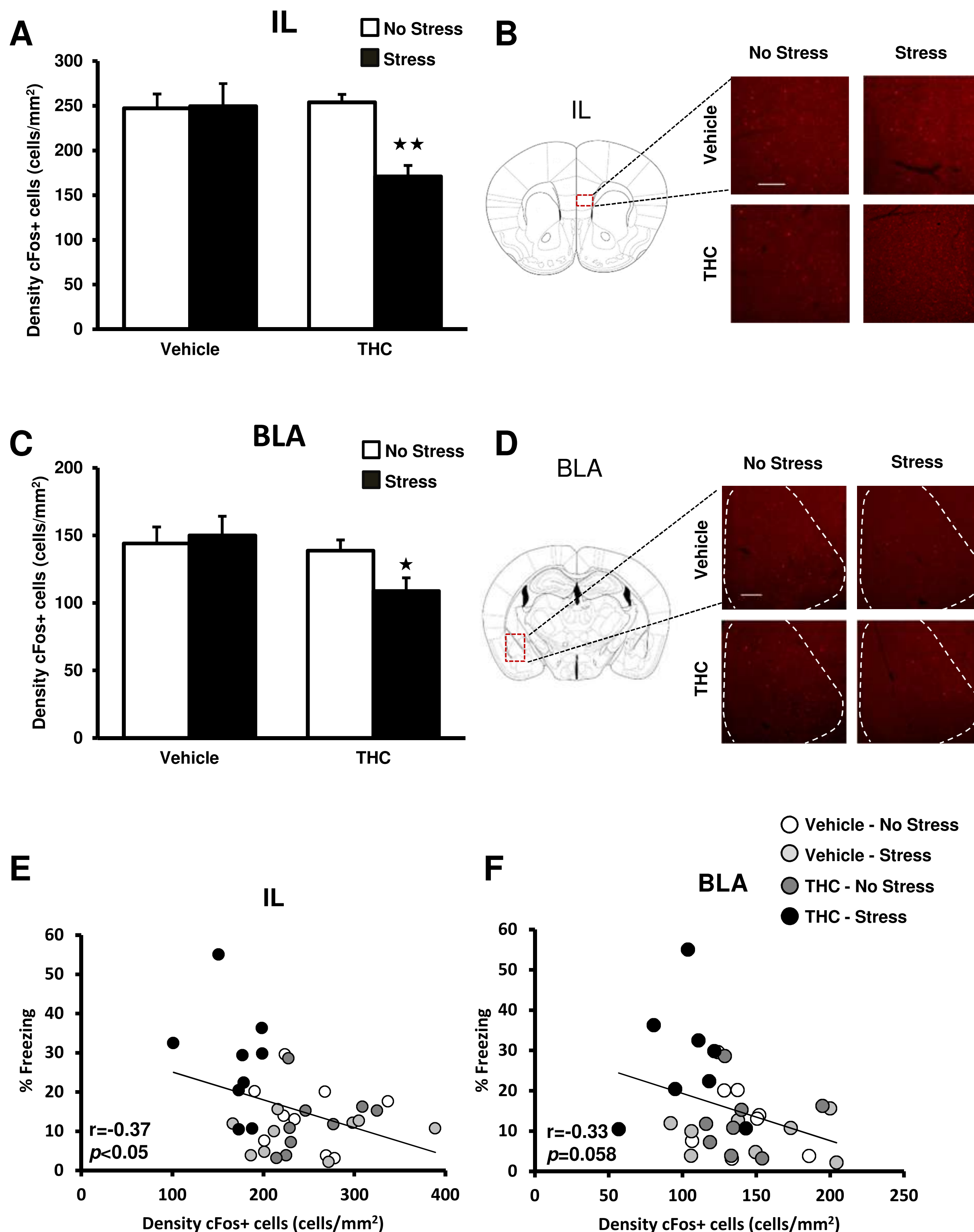

Fig. 4. Impaired fear extinction in adulthood induced by concomitant adolescent THC and stress exposure is associated with a reduced activity of infralimbic prefrontal cortex and basolateral amygdala. (A,C) Density of cFos-expressing cells in (A) IL and (C) BLA 2 h after the last cue extinction session of adult mice exposed to THC, stress, or concomitant THC/stress during adolescence (n = 8–9 mice per group). (B,D) Schematic representation of the anatomical location of (B) IL and (D) BLA adapted from Paxinos and Franklin's stereotaxic atlas (Paxinos and Franklin, 2001), and representative images of both regions obtained by fluorescence microscopy after direct labelling with rabbit polyclonal antiserum to cFos. (E,F) Correlation between fear memory (freezing values) and (E) IL and (F) BLA activity (density of cFos + cells) after the last cue extinction session. Scale bar represents 100 µm. Data are expressed as mean ± SEM. ★$p < 0.05$; ★★$p < 0.01$ (comparison between THC/stress and THC/non stress mice). IL: infralimbic prefrontal cortex. BLA: basolateral amygdala. (Fisher LSD test).

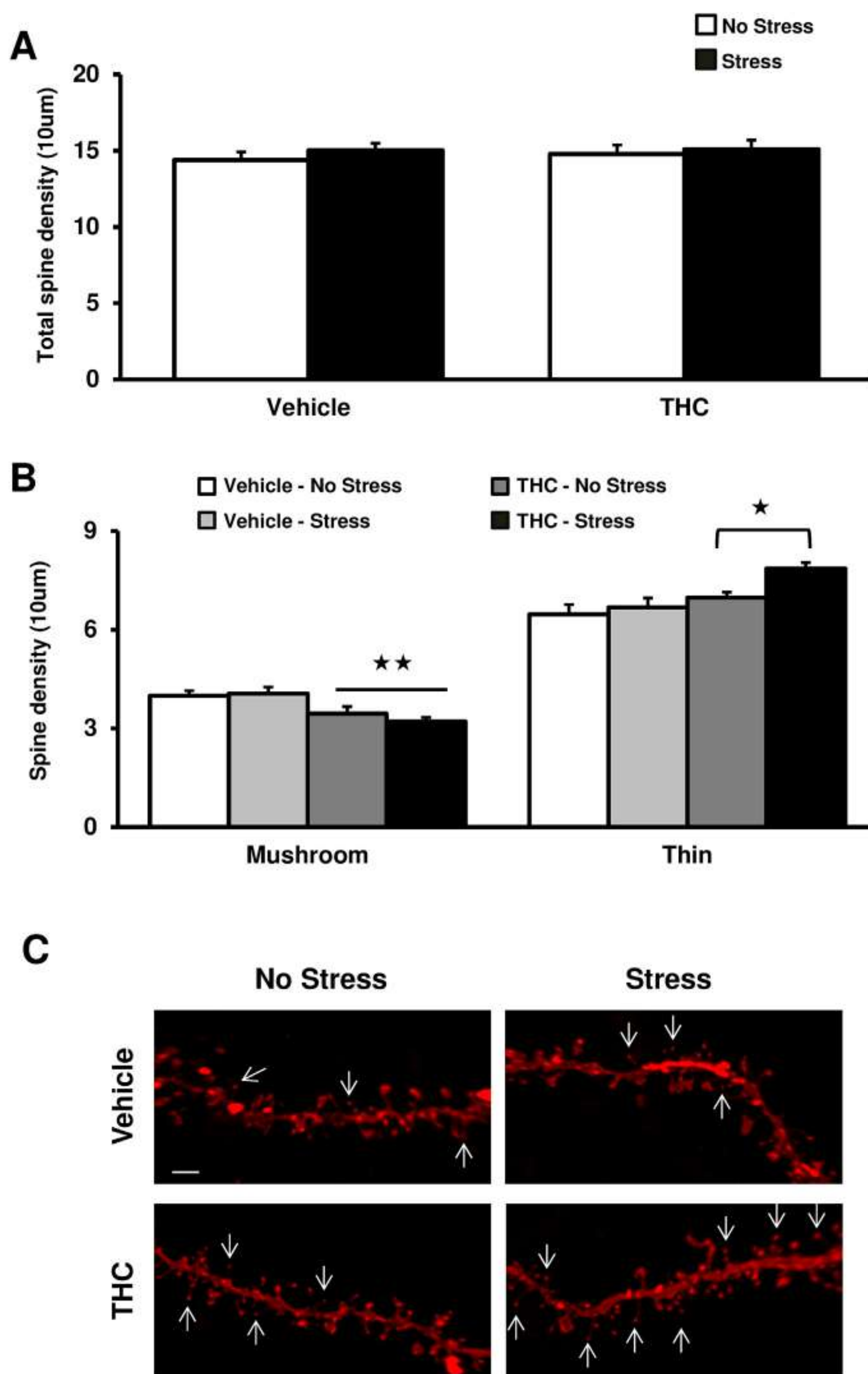

Fig. 5. Impaired fear extinction in adulthood induced by concomitant adolescent THC and stress exposure is associated with structural plasticity alterations in the basolateral amygdala. (A) Overall dendritic spine density, (B) analysis of spine morphology and (C) representative DiOlistics staining of BLA pyramidal neurons after the last cue extinction session of adult mice exposed to THC, stress, or concomitant THC/stress during adolescence (n = 6–8 mice per group). Arrows indicate thin (immature) spines. Scale bar represents 2 µm. Data are expressed as mean ± SEM. ★$p < 0.05$ (comparison between THC/stress and THC/non stress mice); ★★$p < 0.01$ (comparison between THC and vehicle groups). BLA: basolateral amygdala. (Fisher LSD test).

female, but not male, rats in comparison with control animals (Simone et al., 2018b). Fear extinction, which has not been previously evaluated, was not affected in either male or female mice by adolescent THC treatment under our experimental conditions.

Although the exact cause of most psychiatric illnesses is not well known, it is becoming clear that many of these conditions are caused by the combination of genetic and environmental factors or by the association between different environmental events (Caspi and Moffitt, 2006; Rubino and Parolaro, 2016). Stress has a critical role in the development of many psychiatric conditions including trauma-related disorders (Maren and Holmes, 2016). Fear extinction deficits have been reported following exposure to various types of stressors in either animal models (Maren and Holmes, 2016) or humans (Hartley et al., 2014). Thus, adolescent rodents exposed to chronic stress usually show impaired extinction when tested as adults (Judo et al., 2010; Ishikawa et al., 2012; Toledo-Rodriguez et al., 2012; Skelly et al., 2015), although different results have been reported depending on the sex and the modality, chronicity and precise timing of stress exposure during adolescence (Morrissey et al., 2011; McCormick et al., 2013; Schayek and Maroun, 2015; Deng et al., 2017). We found that adolescent stress exposure enhanced anxiety-like behaviour, but did not modify fear conditioning in adult mice. In contrast, concomitant adolescent THC and stress exposure induced an impairment of fear extinction in adulthood without affecting the acquisition of fear memory or general anxiety. Notably, the effect of this THC/stress exposure was age-dependent as revealed by the lack of deficits in fear extinction when the same exposure was performed directly in adulthood. Impaired extinction was not observed by the individual exposition to each factor suggesting synergistic detrimental consequences of adolescent cannabis and stressful events. In this regard, chronic adolescent treatment with

THC induced a deficit in cued fear conditioning only in adult mice with a mutation in disrupted-in-schizophrenia 1 gene (Ballinger et al., 2015), although the influence of this genetic and environmental interaction in fear extinction was not evaluated in this study.

Animals simultaneously exposed to THC and stress during adolescence showed a temporal imbalance of plasmatic corticosterone levels in the adult period as revealed by the increase of this hormone following the cued fear conditioning session. Although the association between glucocorticoids and fear extinction is complex, this enhancement of the hypothalamic-pituitary-adrenal (HPA) axis activity could participate in the extinction deficits and changes in structural plasticity revealed in our study. Thus, a single exposure to immobilization stress in adult male mice activates the HPA axis by increasing corticosterone plasma levels (Andero et al., 2011). A week after this stress exposure mice present impaired cued-fear extinction and enhanced levels of corticosterone after both fear acquisition and fear extinction (Sawamura et al., 2016). Moreover, acute corticosterone administration in adult rats induced dendritic hypertrophy of BLA neurons and enhanced anxiety 12 days after the treatment (Mitra and Sapolsky, 2008; Kim et al., 2014).

IL and BLA are key structures involved in the neurobiological substrate underlying fear extinction (Sierra-Mercado et al., 2011). IL facilitates the activation of the subpopulation of BLA neurons directly involved in fear extinction (Herry et al., 2008). Several studies evaluating immediately-early gene (IEG) expression conclude that impaired fear extinction is associated with reduced activity of the cortico-amygdala circuit (Herry and Mons, 2004; Holmes and Singewald, 2013), while increased IEG levels in those brain areas is related to a complete extinction of conditioned fear (Herry and Mons, 2004; Flores et al., 2014). Notably, our data reveal a reduced cFos expression in the IL and the BLA in adult mice simultaneously exposed to THC and stress during the adolescent period suggesting the existence of a long-lasting dysregulation of the fear circuit. In contrast, PL activity was not modified consistent with a role for this brain area in fear expression, but not extinction (Sierra-Mercado et al., 2011). Besides these modifications on brain region activity, we found structural plasticity changes in pyramidal neurons of the BLA in adulthood as a consequence of adolescent THC treatment, alone or in combination with stress. A decrease of mushroom (matures) dendritic spines was revealed in mice previously exposed to THC congruent with previous studies showing reduced spine density in the dentate gyrus of the hippocampus (Rubino et al., 2009b) and the prefrontal cortex (Rubino et al., 2015) of adult male and female rats, respectively. Interestingly, simultaneous adolescent THC and stress exposure induced an increase of thin (immatures) spines in pyramidal neurons of the BLA in adult mice. These dendritic morphology modifications in BLA neurons associated with the decreased activity of this nucleus could be responsible of the deficits in the extinction of aversive memories. In agreement, fear extinction deficits following acute stress have been related to dendritic retraction in pyramidal neurons of both BLA (Maroun et al., 2013) and IL (Moench et al., 2016).

In summary, our data show lasting neurobiological changes associated with resistance to fear extinction due to concomitant adolescent THC and stress exposure. This study identifies a potential social group highly vulnerable to develop anxiety disorders characterized by pathological fear after cannabis consumption, which contains multitude bioactive compounds (ElSohly et al., 2017). Although our study was performed using pure THC, significantly higher THC concentrations in cannabis derived extracts over the years have been reported (Cascini et al., 2012; ElSohly et al., 2016), which could cause an increase of the total amount of THC consumed. This increase poses higher risk of cannabis use, particularly among adolescents (ElSohly et al., 2016). Taking into account the high rate of cannabis intake during this period which usually entails stressful events, the combination of both factors may increase the risk to suffer anxiety disorders in the adult period. Indeed, the exposure to stressful events during adolescence that often occur in cannabis consumers may represent an important risk factor to suffer anxiety disorders in adulthood.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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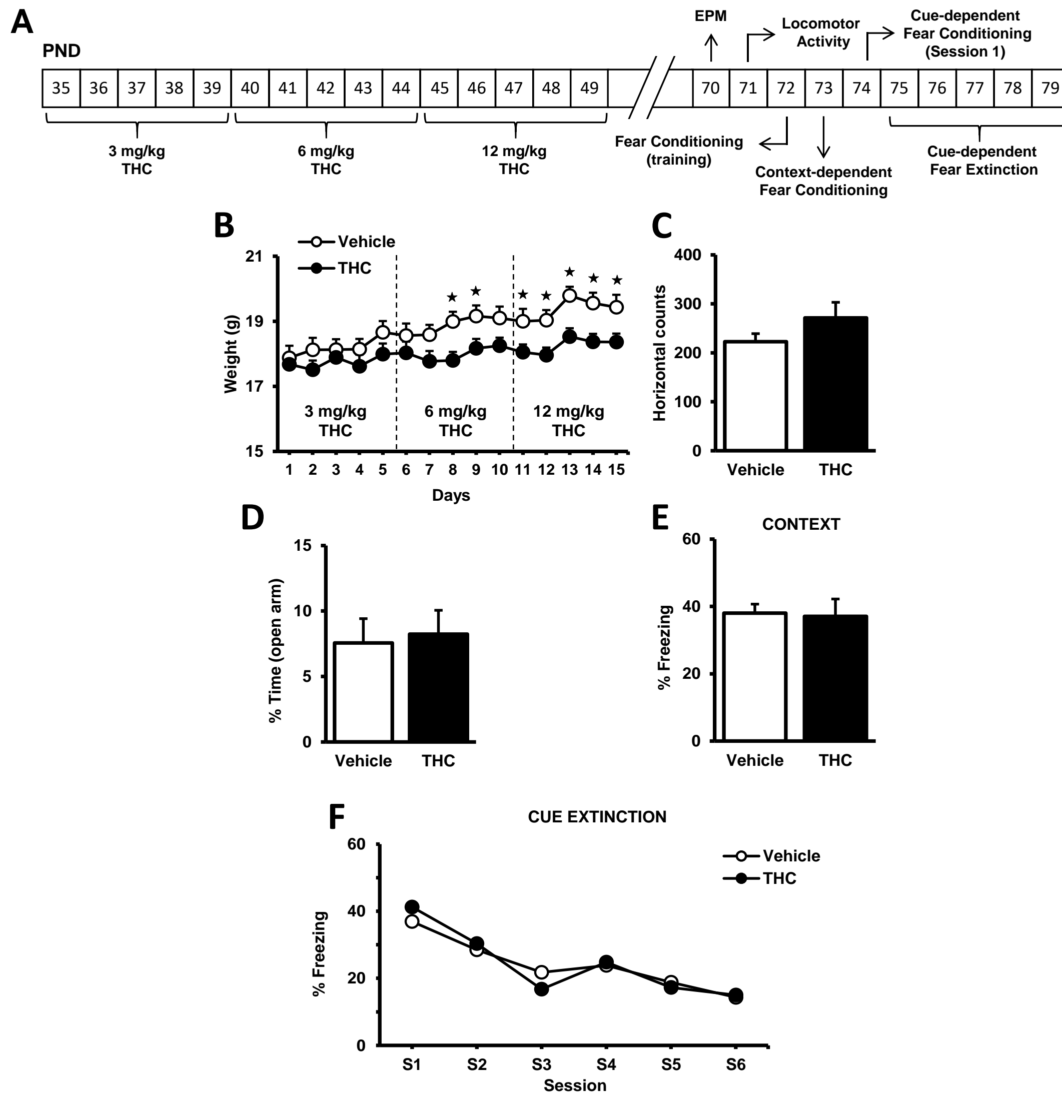

Figure S1. Adolescent THC treatment does not modify fear memory processing in adult female mice. (A) Schematic representation of the experimental design for (B-F). (B) Body weight of adolescent mice during the 15 days of treatment with THC (PND 35-39: 3 mg/kg, PND 40-44: 6 mg/kg, and PND 45-49: 12 mg/kg) or vehicle. (C) Locomotor activity expressed as horizontal counts of adult mice after adolescent THC or vehicle treatment. (D) Anxiety-like behaviour in the EPM expressed as the percentage of time spent in the open arm of mice after adolescent THC or vehicle treatment. (E) Freezing levels scored during contextual fear conditioning in mice treated with THC or vehicle during adolescence. (F) Time course of the freezing levels scored during cued fear extinction trials in adult mice exposed to THC or vehicle during adolescence. Data are expressed as mean ± SEM (n = 10 mice per group). ★$p<0.05$ (comparison between THC and vehicle). PND: postnatal day. EPM: elevated plus maze. S: Session.

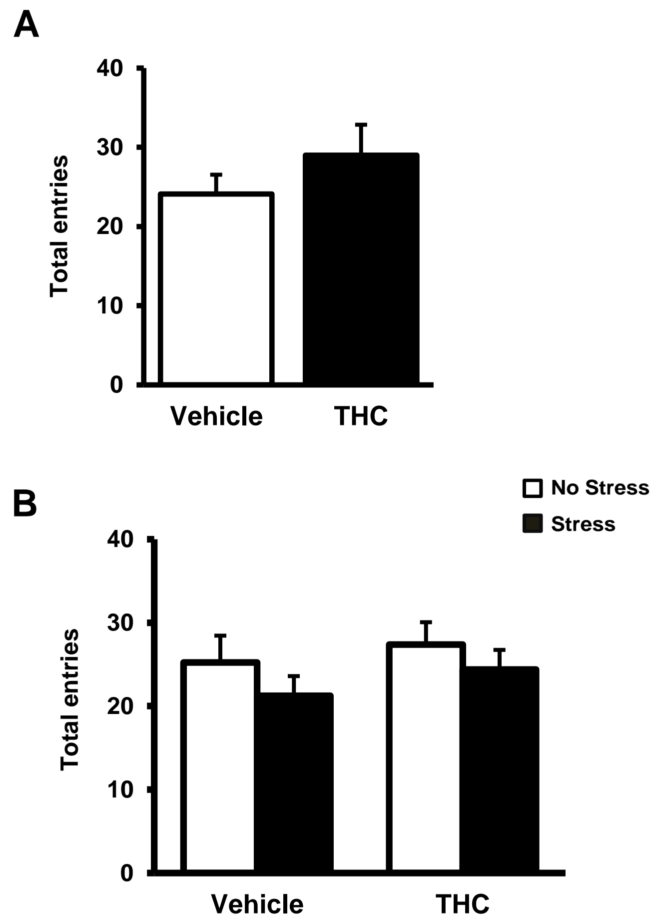

Figure S2. Total number of entries in the elevated plus maze of (A) male mice treated with THC or vehicle during adolescence (n = 10 mice per group). (B) Total number of entries of male mice treated with THC or vehicle and exposed to stress during adolescence (n = 8 mice per group). Data are expressed as mean ± SEM.

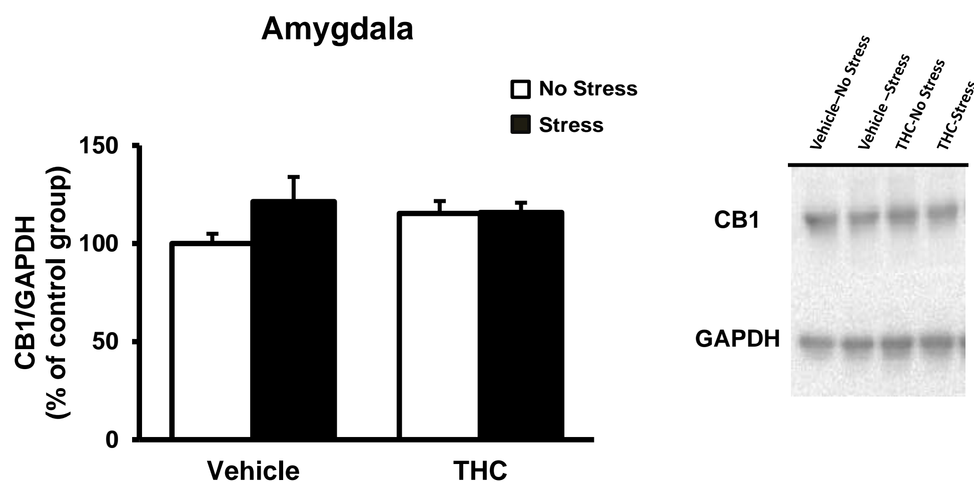

Figure S3. CB1 receptor levels in adult mice treated with THC or vehicle and exposed to stress during adolescence. Immunoblot analysis was used to determine total CB1 receptor levels in the amygdala. Displayed are representative blots showing CB1 receptor expression, as well as GAPDH levels as a loading control for each corresponding sample. Data are expressed as mean ± SEM of the densitometric value for CB1 receptors related to GAPDH, as a percentage of the Vehicle-No stress group (n = 5-6 mice per group).

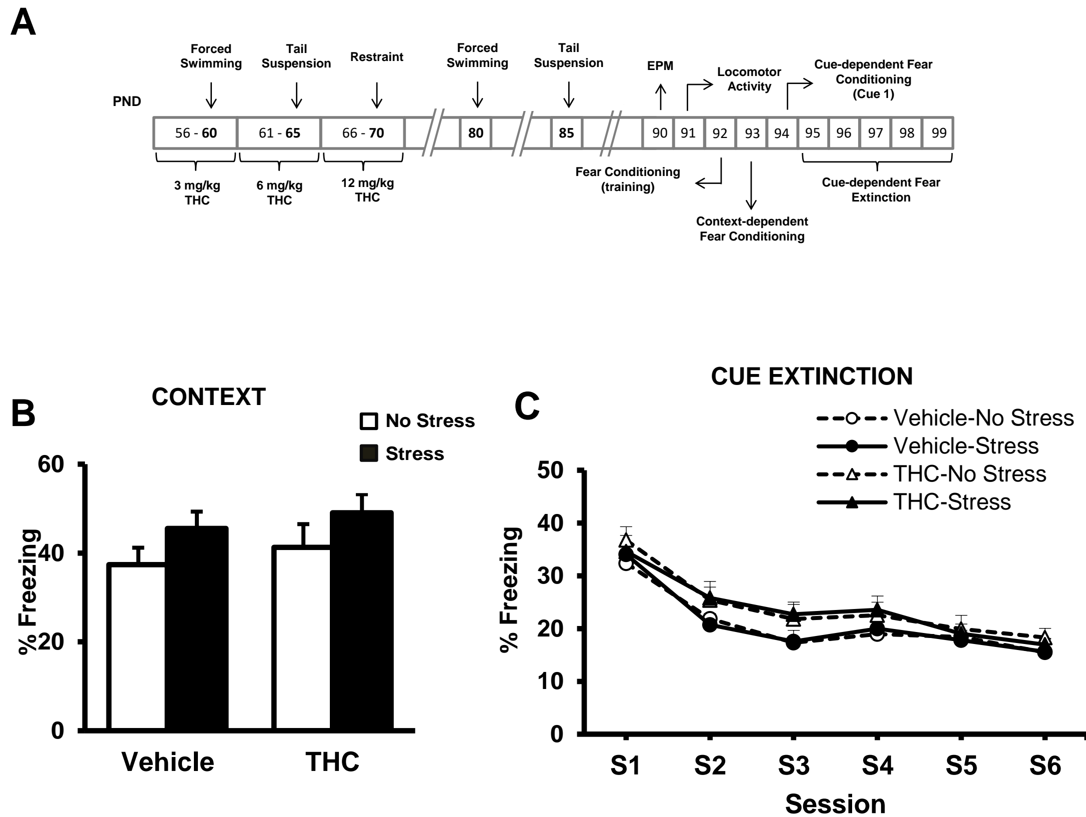

Figure S4. THC and stress exposure during adulthood does not modify the extinction of cued fear memories. (A) Schematic representation of the experimental protocol. Freezing levels scored during the (B) context-dependent fear conditioning and (C) cue-dependent fear conditioning (S1) of mice treated with THC and exposed to stress. (C) Time course of freezing behaviour scored during cued fear extinction trials of mice treated with THC and exposed to stress (*n* = 8 mice per group). Data are expressed as mean ± SEM.

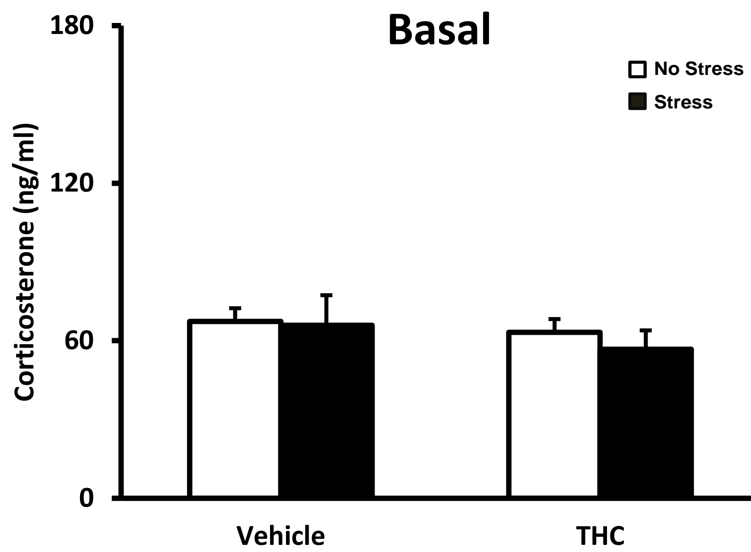

Figure S5. Corticosterone levels during the resting phase of the experimental design of adult mice exposed to THC and stress during adolescence (*n* = 7-8 mice per group). Data are expressed as mean ± SEM.

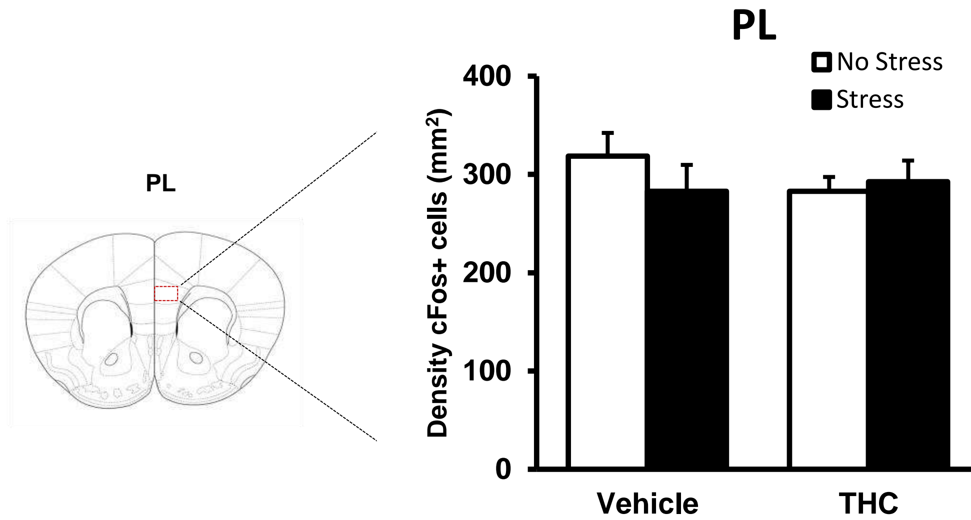

Figure S6. cFos expression in the prelimbic prefrontal cortex of adult mice exposed to THC and stress during adolescence (*n* = 6 mice per group). Schematic representation of the anatomical location of the prelimbic prefrontal cortex adapted from Paxinos and Franklin stereotaxic atlas. Data are expressed as mean ± SEM.

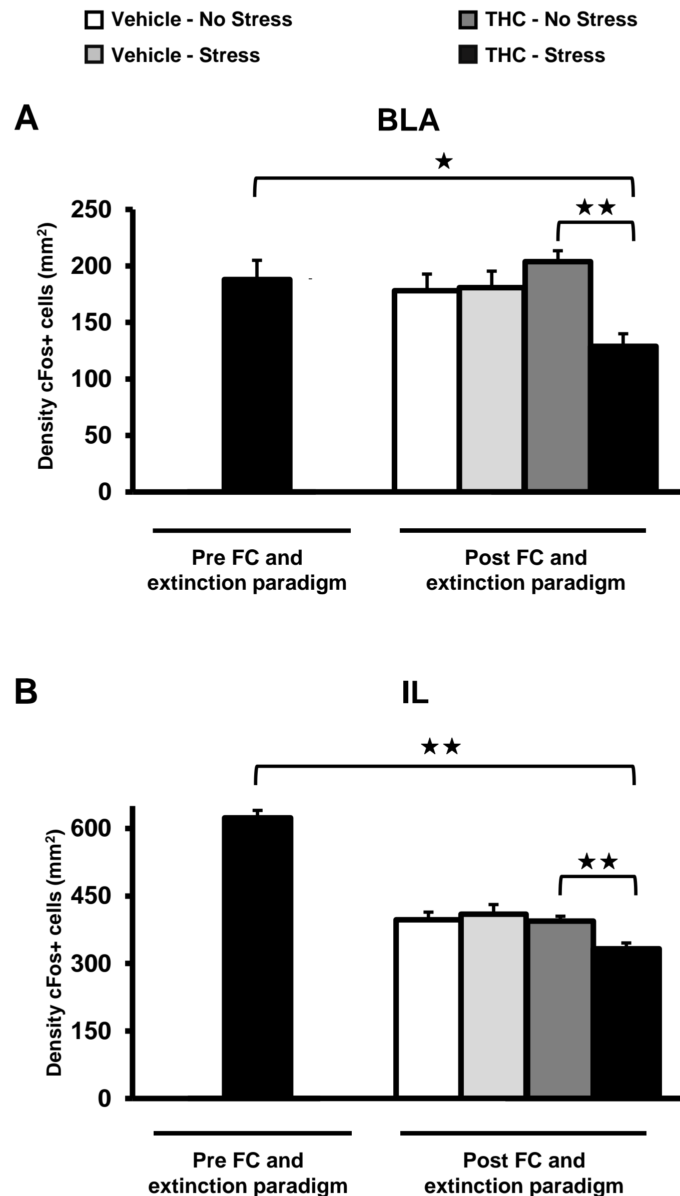

Figure S7. cFos expression is higher in adult animals exposed to THC and stress during the adolescence that do not undergo fear conditioning/extinction, in comparison with mice that followed the behavioural paradigm. (A,B) Density of cFos-expressing cells in (A) BLA and (B) IL before fear conditioning/extinction and 2 h after the last cue extinction session of adult mice exposed to THC, stress, or concomitant THC/stress during adolescence (n = 6-7 mice per group). Data are expressed as mean ± SEM. ★$p<0.05$ (comparison between THC/stress mice before and after FC conditioning/extinction paradigm in (A)); ★★$p<0.01$ (comparison between THC/non-stress and THC/stress mice in (A) and (B); comparison between THC/stress mice before and after FC conditioning/extinction paradigm in (B)). IL: infralimbic prefrontal cortex. BLA: basolateral amygdala. FC: fear conditioning.

Article 2

Amygdalar CB2 cannabinoid receptor mediates fear extinction deficits promoted by orexin-A/hypocretin-1

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MAIN CONCLUSIONS OF THE ARTICLE

In this article, we deciphered an underlying mechanism of OXA-induced impaired fear extinction, thus identifying 2-AG and CB2R as the main endocannabinoid components responsible for this effect. In addition, we found that CB2Rs located in the amygdala are the main effectors of such response, which seem to be present in microglial cells.

My contributions in the present article are the participation in the experimental design, as well as in the behavioural and biochemical experiments, excluding the quantification of endocannabinoid levels. I was also involved in data curation, statistical analysis, writing the original draft and the corresponding manuscript revisions, and the dissemination of results.

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Amygdalar CB2 cannabinoid receptor mediates fear extinction deficits promoted by orexin-A/hypocretin-1

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Orexin-A (PubChem CID: 92131430)
O7460 (PubChem CID: 132285144)
Rimonabant (PubChem CID: 104849)
AM630 (PubChem CID: 4302963)
JWH133 (PubChem CID: 6918505)
PLX5622 (PubChem CID: 52936034)

ABSTRACT

Anxiety and stress disorders are often characterized by an inability to extinguish learned fear responses. Orexins/hypocretins are involved in the modulation of aversive memories, and dysregulation of this system may contribute to the aetiology of anxiety disorders characterized by pathological fear. The mechanisms by which orexins regulate fear are unknown. Here we investigated the role of the endogenous cannabinoid system in the impaired fear extinction induced by orexin-A (OXA) in male mice. The selective inhibitor of 2-arachidonoylglycerol (2-AG) biosynthesis O7460 abolished the fear extinction deficits induced by OXA. Accordingly, increased 2-AG levels were observed in the amygdala and hippocampus of mice treated with OXA that do not extinguish fear, suggesting that high levels of this endocannabinoid are related to poor extinction. Impairment of fear extinction induced by OXA was associated with increased expression of CB2 cannabinoid receptor (CB2R) in microglial cells of the basolateral amygdala. Consistently, the intra-amygdala infusion of the CB2R antagonist AM630 completely blocked the impaired extinction promoted by OXA. Microglial and CB2R expression depletion in the amygdala with PLX5622 chow also prevented these extinction deficits. These results show that overactivation of the orexin system leads to impaired fear extinction through 2-AG and amygdalar CB2R. This novel mechanism could be of relevance for the development of novel potential approaches to treat diseases associated with inappropriate retention of fear, such as post-traumatic stress disorder, panic anxiety and phobias.

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]− 1 H-indol-3-yl](4-methoxyphenyl)methanone; ANOVA, analysis of variance; BLA, basolateral amygdala; CB1R, cannabinoid receptor-1; CB2R, cannabinoid receptor-2; CSF1R, colony stimulating factor 1 receptor; CX3CR1, C-X3-C motif chemokine receptor 1; DAGL, diacylglycerol lipase; DMSO, dimethyl sulfoxide; ECS, endocannabinoid system; eGFP, enhanced green fluorescent protein; FAAH, fatty acid amide hydrolase; GABA, γ-aminobutyric acid; Iba1, ionized-calcium binding adapter 1; JZL184, 4-[Bis(1,3-benzodioxol-5-yl) hydroxymethyl]− 1-piperidinecarboxylic acid 4-nitrophenyl ester; JWH133, (6aR,10aR)− 3-(1,1-Dimethylbutyl)− 6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6 H-dibenzo[*b*,*d*]pyran; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; O7460, 2-[(fluoromethylphosphinyl)oxy]− 1-[(1-methylethoxy)methyl]ethyl ester, 9Z-octadecenoic acid; OX1R, orexin receptor-1; OXA, orexin-A; PLX5622, 5-fluoro-N-[6-fluoro-5-[(5-methyl-1 H-pyrrolo[2,3-*b*]pyridin-3-yl)methyl]− 2-pyridinyl]− 2-methoxy-3-pyridinemethanamine; PTSD, post-traumatic stress disorder; URB597, cyclohexylcarbamic acid 3′-(Aminocarbonyl)-[1,1′-biphenyl]− 3-yl ester.

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1. Introduction

Orexin-A/hypocretin-1 (OXA) and Orexin-B/hypocretin-2 (OXB) are neuropeptides of the lateral hypothalamus that project throughout the brain [1,2] and bind two G protein-coupled receptors, orexin receptor-1 (OX1R) and (OX2R) [1,2]. The orexin system is involved in diverse physiological functions including fear regulation [3,4], consistent with the existence of orexin neuronal projections to several limbic areas [5].

Pharmacological blockade or genetic deletion of OX1R impaired contextual and cued fear conditioning [6–9] in rodents. Moreover, OX1R antagonism facilitated fear extinction consolidation [8,10,11], while OXA administration impaired this response [8]. Accordingly, the activity of orexin neurons was negatively correlated with successful extinction of conditioned fear in rats [12]. Reactivity to CO2 was significantly predictive of orexin activity in the lateral hypothalamus, and in turn high orexin activity was associated with poor extinction [13]. In humans, several studies have also described a relationship between orexins and fear and anxiety. Individuals with narcolepsy, a condition associated with a loss of orexin neurons [14], showed reduced amygdala activity and failed to acquire fear memory during aversive conditioning [15]. Patients with panic anxiety have elevated levels of OXA in the cerebrospinal fluid (CSF) [16]. However, a clinical study showed a reduction of OXA levels in the CSF and plasma of combat veterans with chronic PTSD and these levels were negatively correlated with PTSD severity [17]. Recently, an interaction between genetic polymorphisms of the OX1R and ghrelin genes was shown to affect PTSD symptom severity [18].

The endocannabinoid system (ECS), composed of two main receptors, the cannabinoid type-1 and type-2 receptors (CB1R and CB2R, respectively), their ligands, i.e. the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes involved in endocannabinoid metabolism [19] is an important neuromodulatory system crucial for appropriate fear extinction [20]. AEA through CB1R activation in the basolateral amygdala (BLA) facilitates fear extinction [21,22]. However, the role played by 2-AG in this response is less evident, and it has been suggested that an optimal level of this endocannabinoid is required for appropriate processing of fear responses [23, 24]. Several reports have described the existence of functional interactions between orexins and 2-AG, mainly in the regulation of nociception, reward and food intake [25,26]. However, whether the ECS is part of the neurobiological substrates underlying the modulation that orexins exert on fear remains to be clarified.

In this study, we investigated the participation of the ECS in the fear extinction deficits induced by orexin-A. Understanding the neurobiological mechanisms involved in these effects is essential to identify novel targets for the treatment of anxiety disorders characterized by pathological fear.

2. Material and methods

2.1. Animals

Experiments were performed using male C57BL/6 J mice (Jackson Laboratories) and the recently characterized eGFP-CB2R mice (generated by Dr. Julián Romero and Dr. Cecilia J. Hillard) and their wild-type controls (8–12 weeks old) [27]. eGFP-CB2R mice result in the expression of the enhanced green fluorescent protein (eGFP) reporter gene under the control of the endogenous mouse CB2R promoter. eGFP-CB2R mice were backcrossed for at least five generations to C57BL/6 J mice. Mice were housed in cages holding a maximum of 5 mice per cage and maintained in a temperature (21.1 ± 1 °C)- and humidity (55 ± 10%)-controlled room. Mice implanted with unilateral or bilateral cannulae were individually housed to avoid cannulae shifting or removal. Food and water were available ad libitum. Lightning was maintained in 12 h light/dark cycles (light on at 8:00 AM and off at 8:00 PM). All experiments were performed during the light phase. Mice were handled daily for 3 days before the beginning of the experiments. All behavioural experiments were performed under blind conditions. Experimental procedures were conducted in the animal facilities of Universidad Francisco de Vitoria in Madrid, Spain, in accordance with the guidelines of the European Communities Directive 2010/63/EU and the Spanish Regulations RD 1201/2005 and 53/2013 regulating animal research and approved by the local ethical committee (CEEA-UFV).

2.2. Drugs

OXA (synthesized by Dr. David Andreu, Proteomics and Protein Chemistry, UPF, Barcelona) was dissolved in physiological saline and administered by intracerebroventricular (i.c.v.) route at 0.75nmol·μl^{-1} or intra-BLA (0.375nmol/0.5 μl/side). This dose was based on previous studies [8]. The FAAH inhibitor URB597 (3 mg·kg^{-1}) (Sigma) and the MAGL inhibitor JZL184 (8 mg·kg^{-1}) (Tocris), dissolved in physiological saline and in a solution of 15% dimethyl sulfoxide (DMSO), 5% Tween and 80% saline respectively, were administered by intraperitoneal (i.p.) route (5 ml·kg^{-1} body weight). The DAGL inhibitor O7460 (synthesized by Dr. Vincenzo Di Marzo, Pozzuoli, Italy) was dissolved in 10% DMSO and 90% saline, and a volume of 1 μl was administered i.c.v. at 1 μg·μl^{-1}. The CB1R and CB2R antagonists, rimonabant (0.1, 0.5 and 1 mg·kg^{-1}) (Tocris) and AM630 (0.5, 3 and 5 mg·kg^{-1}) (Sigma) respectively, were administered i.p. (10 ml·kg^{-1} body weight). Rimonabant was dissolved in a solution of 5% ethanol, 5% cremophor and 90% saline. AM630 was dissolved in a solution of 10% DMSO, 10% Tween 80% and 80% saline for i.p. and in DMSO/saline (2:1) for intra-BLA infusion (3 μg/0.5 μl/side). The CB2R agonist JWH133 (2 mg·kg^{-1}) (Tocris) was dissolved in a solution of 10% DMSO, 10% Tween 80% and 80% saline, and administered by i.p. route (10 ml·kg^{-1} body weight). This dose was based on previous studies [28]. Ketamine hydrochloride (7.5 mg·kg^{-1}) and dexmedetomidine hydrocloride (0.5 mg·kg^{-1}) were mixed and dissolved in saline, and administered i.p. (6 ml·kg^{-1} body weight).

2.3. Behavioural experiments

2.3.1. Contextual fear conditioning and extinction

Mice were contextually fear-conditioned as performed in preceding experiments and based on previous results of our group [8,10]. The test chamber (LE116, Panlab) was made with black methacrylate walls and a transparent front door. This chamber (25 ×25×25 cm) was located inside a soundproof module with a ventilation fan in order to provide a background noise and attenuate surrounding sounds. The chamber floor was constructed of parallel stainless-steel bars of 2 mm of diameter spaced at 6 mm intervals and was connected to a scrambled shock generator (LE100–26 module, Panlab). A high-sensitivity weight transducer (load cell unit) was used to record and analyse the signal generated by the animal movement intensity. Experimental software PACKWIN V2.0 automatically calculated the percentage of immobility time for each experimental phase. Before each trial, the chamber floor and walls were cleaned with 70% ethanol and then water to avoid olfactory cues. On the conditioning session, mice were individually placed in the chamber during 180 s before the exposure to the first unconditioned stimulus (US) in the absence of any stimulus to habituate mice to the new environment. After the US (0.7 mA footshock for 1 s), mice were left for 60 s to associate the US with the conditioned stimulus. A second shock was given and then mice remained in the chamber for additional 60 s. Fear extinction trials (E1-E5) were performed 24, 48, 72, 96 and 120 h after the conditioning day.

To study the consolidation of fear extinction, pharmacological treatments were administered immediately after the extinction session, except OXA which was administered 20 min later. Fear memory was assessed as the percentage of time that mice spent freezing during the first 3 min of each 5-minutes trial. Freezing behaviour, a rodent's natural response to fear, was automatically evaluated and defined as complete lack of movement, except for breathing for more than 800 ms.

Data from fear extinction were expressed as percentage of freezing behaviour and as area under the curve (AUC). AUC was calculated by using a standard trapezoid method, $AUC = [0.5 \times (B1 + B2) \times h] + [0.5 \times (B2 + B3) \times h] + \ldots [0.5 \times (Bn+ Bn+1) \times h]$, where Bn were the percentage of freezing behaviour for each mouse and h was the time (days) passed between the consecutive measurements. Biochemical studies were carried out after the second extinction trial.

2.3.2. Locomotor activity

Locomotor activity was evaluated as previously reported [8]. Changes in locomotor activity were assessed by using locomotor activity boxes (27 ×27×21 cm, Cibertec). Mice were placed in locomotor cages with low luminosity. Activity was measured as the total number of infrared beams crossed by the animal every 5 min for 20 min.

2.4. Stereotaxic surgery and infusion procedure

Surgical procedures for i.c.v. infusion of OXA and intra-BLA administration of AM630 and OXA were performed as previously reported [8].

2.4.1. Intracerebroventricular infusion

Mice were anesthetized with a ketamine/dexmedetomidine mixture and positioned in a stereotaxic frame (KOPF Instruments). A small hole was drilled on the left or the right side of the skull randomly and an unilateral cannula (26 gauge, Plastics One) was implanted vertically into the left/right lateral ventricle according to Paxinos and Franklin [29] (from bregma: AP, −0.20 mm; ML, +/-1.00 mm; DV, 2.25 mm). The cannula was subsequently fixed to the skull with dental cement and closed with a dummy cap (33-gauge internal cannula, Plastics One). Mice were housed individually and allowed 3 days of post-operative recovery before behavioral experiments began. Microinjection procedure of OXA (0.75 nmol·µl) was performed by connecting the cannula of freely moving mice to an injection cannula (33-gauge internal cannula, Plastics One) connected to a polyethylene tubing (PE-20, Plastics One) attached to a 10 µl microsyringe (Model 1701 N SYR, Cemented NDL, 26 ga, 2 in, point style 3, Hamilton Company). A total volume of 1 µl was injected at a constant rate of 1 µl·min by using a microinfusion pump (Harvard Apparatus, Holliston). The injection cannula was removed 1 min after OXA infusion to prevent drug reflux. After completion of the behavioral experiments, 0.05% methylene blue solution (Sigma) was infused to check the correct position of the cannula. Only those mice with correct injection sites were included in the statistical analysis.

2.4.2. Intra-amygdala microinjection

Stereotaxic surgery was performed as explained above. Bilateral guide cannulae (26 gauge, Plastics One) were implanted vertically into the basolateral amygdala (BLA) according to Paxinos and Franklin [29] (from bregma: AP, −1.70 mm; ML, +/-3.35 mm; DV, −3.00 mm). Microinjections of AM630 (3 µg/0.5 µl/side) and OXA (0.375 nmol//0.5 µl/side) were performed by inserting an injection cannula (33 gauge, Plastics One) into the guide cannula, which extended 1 mm beyond to reach the BLA. Drugs and vehicles were delivered at a constant rate of 0.5 $\mu l \cdot min^{-1}$ during 1 min. Injection cannula was removed from the guide cannula 1 min after infusion to prevent drug reflux. After completion of the behavioral experiments, coronal sections of each brain were stained with toluidine blue and the injection sites were histologically verified to be within the BLA. Only those mice with correct injection sites were included in the statistical analysis.

2.5. Measurement of endocannabinoid levels

Endocannabinoid quantification was performed as previously reported [30]. Tissues were homogenized in 5 vol of chloroform/methanol/Tris–HCl 50 mM (2:1:1) containing 1 pmol of d8-anandamide (AEA) and d5–2-arachidonoylglycerol (2-AG). Deuterated standards were synthesized from d8-arachidonic acid and ethanolamine or glycerol, or from d4-ethanolamine. Homogenates were centrifuged at 13,000 g for 16 min (4 °C), the aqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform/methanol 99:1 by vol. The solutions were then purified by open bed chromatography on silica. Fractions eluted with chloroform/methanol 9:1 by vol. (containing AEA and 2-AG) were collected and the excess solvent evaporated with a rotating evaporator, and aliquots analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI–MS) and allowing the separations of 2-AG and AEA. MS detection was carried out in the selected ion monitoring mode using m/z values of 356 and 348 (molecular ion +1 for deuterated and undeuterated AEA), and 384.35 and 379.35 (molecular ion +1 for deuterated and undeuterated 2-AG). Values were expressed as pmol or fmol per mg of lipid extract.

2.6. Quantitative RT-PCR analysis

Amygdala, prefrontal cortex and hippocampus tissues were extracted 10 min after the end of the extinction trial and immediately frozen at −80°C. Total RNA was purified with the RiboPure™ Kit (Invitrogen) for amygdala and prefrontal cortex, and the RNeasy Mini Kit (QIAGEN) for hippocampus, according to the manufacturer's instructions. Reverse transcription was performed with 0.8 µg of total RNA and the SuperScript™ II Reverse Transcriptase (Invitrogen). PCR reactions were conducted using PrimePCR™ Probe Assay (Bio-Rad) to quantify mRNA levels for DAGLα (Unique Assay ID: qMmuCIP0032590), MAGL (Unique Assay ID: qMmuCIP0042348), NAPE-PLD (Unique Assay ID: qMmuCIP0035707), FAAH (Unique Assay ID: qMmuCEP0055480), CB1R (Unique Assay ID: qMmuCEP0038879), CB2R (Unique Assay ID: qMmuCEP0039299) and CX3CR1 (Unique Assay ID: qMmuCEP0058111) using GAPDH expression (Unique Assay ID: qMmuCEP0039581) as endogenous control gene for normalization. PCR assays were carried out with the CFX Connect Real-Time PCR Detection System (Bio-Rad). The fold changes in gene expression of OXA-treated animals in comparison with controls were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Tissue preparation, immunofluorescence and image analysis

Immunofluorescence was performed as previously reported [27].

2.7.1. Tissue preparation for immunofluorescence

Mice were deeply anesthetized 30 min after the extinction trial by i.p. injection of the ketamine/dexmedetomidine solution and fixed by intracardiac perfusion with cold phosphate buffer saline (PBS) followed by freshly prepared cold 4% paraformaldehyde. Then, the brain was post-fixed overnight in the same fixative and dehydrated by sequential transfer to 15% and 30% sucrose solutions. Coronal frozen sections of 30 µm thickness were obtained in a cryostat from 0.82 to −1.82 mm relative to bregma for BLA, from 1.98 to 1.54 mm relative to bregma for prefrontal cortex and from −1.46 to −2.18 mm relative to bregma for hippocampus. Brain sections were preserved in cryoprotective solution until use.

2.7.2. Immunofluorescence

Floating brain sections were washed with tris buffer saline (TBS) before overnight incubation at 4 °C with the designated primary antibodies diluted in TBS containing 1% bovine serum albumin (BSA, Sigma) and 1% Triton X-100 (Sigma). Antibodies used in this study were chicken antibody against green fluorescent protein (GFP) (1:1500, Abcam) and rabbit polyclonal antibody to Iba1 (1:1000, Wako). After primary antibody incubations, sections were washed three times in TBS followed by incubations with designed secondary antibodies at 37 °C for

2 h. Secondary antibodies used in this study were Alexa 488-conjugated goat anti-chicken IgY and Alexa 555-conjugated goat anti-rabbit IgG (both from Invitrogen). Slices were washed three times in TBS, mounted on subbed slides, air dried, and coverslipped using Fluoromount-G (Invitrogen).

2.7.3. Image analysis

The stained sections were analysed at 10 x objective using the upright microscope Nikon 90i (Nikon and Axioimager M2, Zeiss) equipped with a DXM 1200 F camera. Images (1024 × 1024 pixels) were obtained by using two different laser lines (488 and 561 nm) and further analysed in ImageJ software. GFP_{+} cells were counted (cells per area) in coronal sections of BLA (from −0.82 to −1.82 mm relative to bregma), prefrontal cortex (from 1.98 to 1.54 mm relative to bregma) and hippocampus (from −1.46 to −2.18 mm relative to bregma). Colocalization of GFP with microglial cells was quantified using the ImageJ manual particle counting option. The option "freehand selections" was used to limit the area of BLA and the microglial soma perimeter. For prefrontal cortex analysis, a 540 µm side square region of interest (ROI) was delimited for quantification. Four to six images per brain area of each animal were analysed.

2.8. Microglial depletion through chow treatment

The colony stimulating factor 1 receptor (CSF1R) inhibitor PLX5622 was provided by Plexxikon (Plexxikon Inc) and formulated in AIN-76A chow at dose of 1200 parts per million (Research Diets). Blockade of maintained CSF1R induces continuous microglial depletion. C57BL/6 mice received PLX5622 or control chow for 4 days after stereotaxic surgery recovery and throughout the behavioural test. The selected dose and duration of PLX5622 treatment were based on previous studies showing 80% microglial depletion with the same dose and similar treatment duration [31].

2.9. Statistical analysis

Comparisons between two groups were assessed by Student's *t* tests. Multiple-group comparisons were performed by one-way or two-way analysis of variance (ANOVA), as appropriate. Repeated-measurement ANOVA was used for serial freezing and locomotion responses. Subsequent Fisher's LSD post-hoc test was only used when ANOVA interaction effects were significant. Pearson correlation coefficient was used to analyse the strength of relationship between two variables. All data were expressed as mean ± SEM. The statistical analysis was performed using Statistica (StatSoft) software. The level of significance was $p < 0.05$ in all experiments.

3. Results

3.1. Impairment of fear extinction induced by OXA is mediated by 2-AG

First, we evaluated the effect of 2-AG and AEA in the extinction of fear memories by inhibiting the activity of monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), the enzymes that degrade 2-AG and AEA respectively, in a contextual fear conditioning paradigm (Fig. 1A). The MAGL inhibitor JZL184 (8 $mg{\cdot}kg^{-1}$, ip), but not the FAAH inhibitor URB597 (3 $mg{\cdot}kg^{-1}$, ip), significantly impaired fear extinction as showed the increase of freezing behaviour and AUC (Fig. 1B,C) when compared to the control group. Locomotor activity was not modified in mice 24 h after acute administration of JZL184 (8 $mg{\cdot}kg^{-1}$, ip) (Fig. S1), demonstrating that the changes observed in freezing behaviour were not due to unspecific effects on locomotion. OX1R activation in response to OXA promotes diacylglycerol (DAG) production which in turn is used by diacylglycerol lipases (DAGL) as a substrate for 2-AG synthesis [26,32]. Interestingly, the pretreatment with the specific DAGL inhibitor O7460 (1 $\mu g{\cdot}\mu l^{-1}$, icv) before OXA (0.75$nmol{\cdot}\mu l^{-1}$, icv) (Fig. 1A) prevented the impairment of fear extinction induced by the neuropeptide (Fig. 1D,E). Taken together, these results suggest that OXA recruits 2-AG to regulate fear extinction.

Next, we studied whether acute OXA infusion, at the same dose that produces impairment of fear extinction, increases 2-AG levels in amygdala, prefrontal cortex and hippocampus, key brain regions related to contextual fear regulation [33]. An enhancement of 2-AG was observed in the amygdala 10 min, but not 30 min, following OXA administration (Fig. 1F). In the prefrontal cortex, 2-AG increased 30 min, but not 10 min, after OXA injection (Fig. 1G). Surprisingly, a decrease of 2-AG was found in the hippocampus 30 min after OXA infusion (Fig. 1H). There were no significant differences between saline and OXA treatment groups at 60 min (Fig. S2), while AEA levels were not modified in any brain area at the different time points analysed (Fig. S3 and S4). All together, these data suggest that OXA induces resistance to fear extinction through increased levels of 2-AG, probably in the amygdala.

3.2. Impaired fear extinction is associated with increased 2-AG levels in the amygdala and hippocampus

To further study the role played by 2-AG in the extinction deficit exerted by OXA, we measured endocannabinoid levels just before and 10 min after the second extinction session in mice treated with saline or OXA (0.75$nmol{\cdot}\mu l^{-1}$, icv) after the first extinction trial (Fig. 2A). As expected, OXA impaired fear extinction in comparison with control mice (Fig. 2B). No changes in 2-AG levels were observed between groups in any brain area before the extinction session (Fig. 2C,D,E). Notably, 2-AG levels increased in the amygdala and hippocampus (Fig. 2C,E), but not in the prefrontal cortex (Fig. 2D), after the extinction session in animals treated with OXA that do not extinguish fear (Fig. 2B). Indeed, a significant correlation between fear memory (freezing values) and 2-AG levels was observed in the amygdala (Fig. 2F). No correlation was found neither in the prefrontal cortex nor in the hippocampus (Fig. S5). OXA did not modify AEA levels analysed before and after the extinction session in any brain region (Fig. S6). These results suggest that an optimal level of 2-AG is required for appropriate processing of fear responses and that high amygdalar and hippocampal 2-AG levels induced by OXA infusion are related to extinction deficits.

3.3. Impairment of fear extinction induced by OXA is associated with increased expression of CB2R in the amygdala

Given the role played by 2-AG in the extinction deficit induced by OXA, we determined the effects of OXA (0.75$nmol{\cdot}\mu l^{-1}$, icv) on gene expression of the endocannabinoid-synthesizing and degrading enzymes, as well as CB1R and CB2R. Brain tissue was removed 10 min after the second extinction session, in OXA- or saline-treated mice after the first session. In the amygdala, OXA infusion increased the mRNA encoding for DAGLα (Fig. 3A), but not MAGL, suggesting that the enhancement of 2-AG previously observed is due to the increase in the synthesis of this endocannabinoid. However, N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression, but not FAAH, was also elevated after OXA treatment (Fig. 3A), indicating that increased expression of AEA biosynthetic enzymes was not sufficient alone to cause here elevation of the levels of the corresponding endocannabinoid. Notably, quantitative RT-PCR analysis showed an increase of CB2R expression (~53%), but not CB1R, in the amygdala of mice treated with OXA that are resistant to fear extinction (Fig. 3A). By contrast, no changes were observed in the expression of any of these genes either in the prefrontal cortex or the hippocampus (Fig. 3B,C).

To study the specific location of the CB2R in the amygdala, we used the recently characterized eGFP-CB2R mice [27], in which the expression of enhanced green fluorescent protein (eGFP) is under the control of the endogenous mouse CB2R promoter. These mice were treated with saline or OXA (0.75$nmol{\cdot}\mu l^{-1}$, icv) after the first session trial, and brain

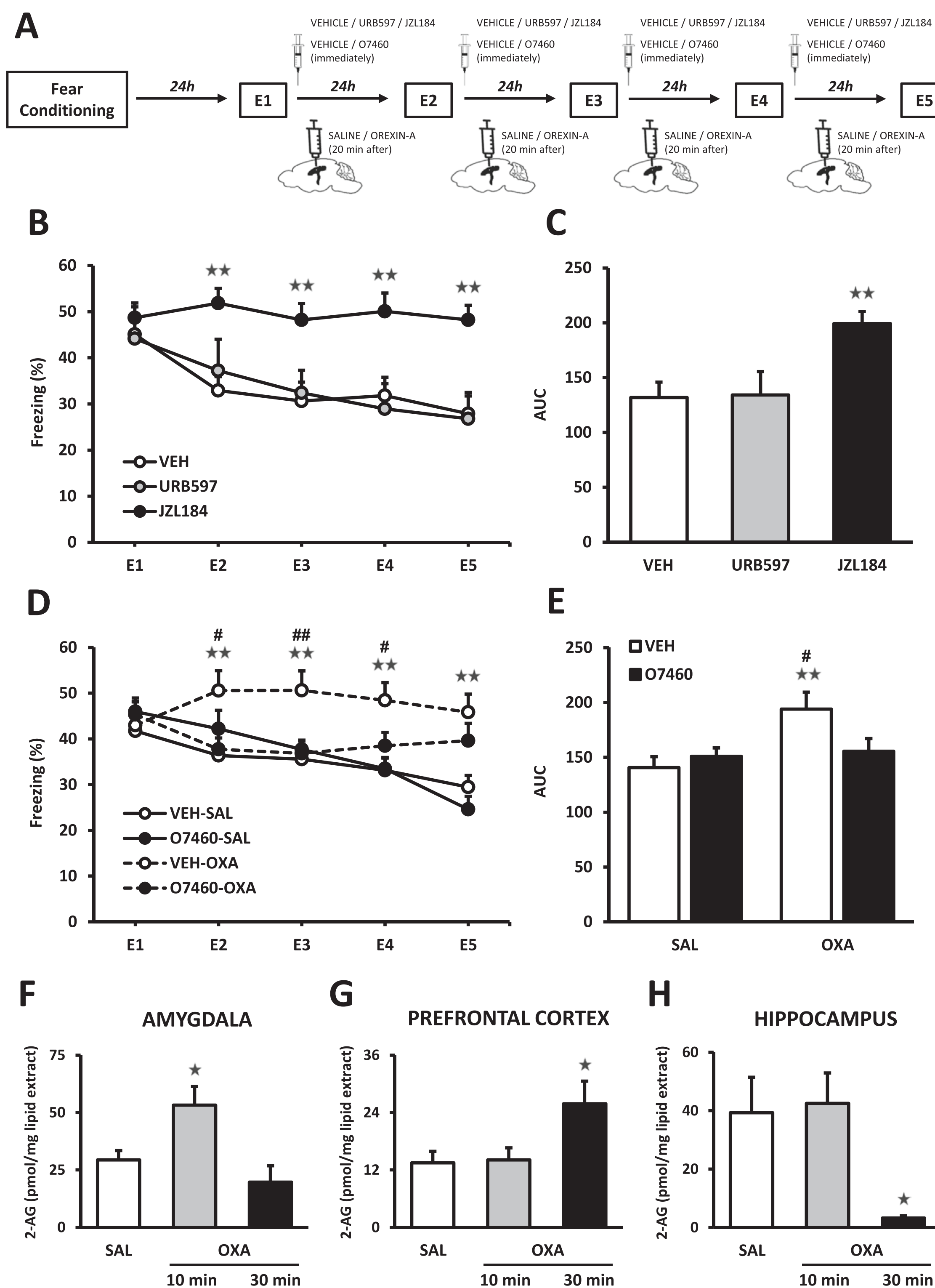

Fig. 1. Impaired fear extinction induced by OXA is modulated by 2-AG. (A) Schematic representation of the experimental design for behavioural tests. (B,C) Time course of the freezing levels during contextual extinction trials (interaction day x treatment: $F_{8,120} = 2.28$, $p < 0.05$) (B) and AUC values (treatment effect: $F_{2,30} = 7.17$, $p < 0.01$) (C) in mice treated immediately after each extinction session with JZL184 (8 mg·kg^{-1}, ip), URB597 (3 mg·kg^{-1}, ip) or VEH (n = 8–3 mice per group). (D,E) Time course of the freezing levels during contextual extinction trials (day x pretreatment x treatment interaction ($F_{4,128} = 3.16$, $p < 0.05$) (D) and AUC values (pretreatment x treatment interaction ($F_{1,32} = 4.11$, $p < 0.05$) (E) in mice treated with O7460 (1 μg·μl, icv) immediately after each extinction session, 20 min before OXA (0.75 nmol·μl^{-1}, icv) infusion (n = 8–10 mice per group). (F,G,H) Levels of 2-AG in amygdala ($F_{2,14} = 6.56$, $p < 0.01$) (F), prefrontal cortex ($F_{2,15} = 4.26$, $p < 0.05$) (G) and hippocampus ($F_{2,15} = 5.56$, $p < 0.05$) (H), in homogenates extracted 10 and 30 min after acute OXA (0.75nmol·μl^{-1}, icv) infusion (n = 5–6 mice per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 (compared with VEH in (B,C), VEH-SAL in (D,E) and SAL in (F,G,H)); #p < 0.05, ##p < 0.01 (comparison with O7460-OXA group). OXA: orexin-A; VEH: vehicle; SAL: saline; E1-E5: extinction trials 1–5; AUC: area under the curve; 2-AG: 2-arachidonoylglycerol.

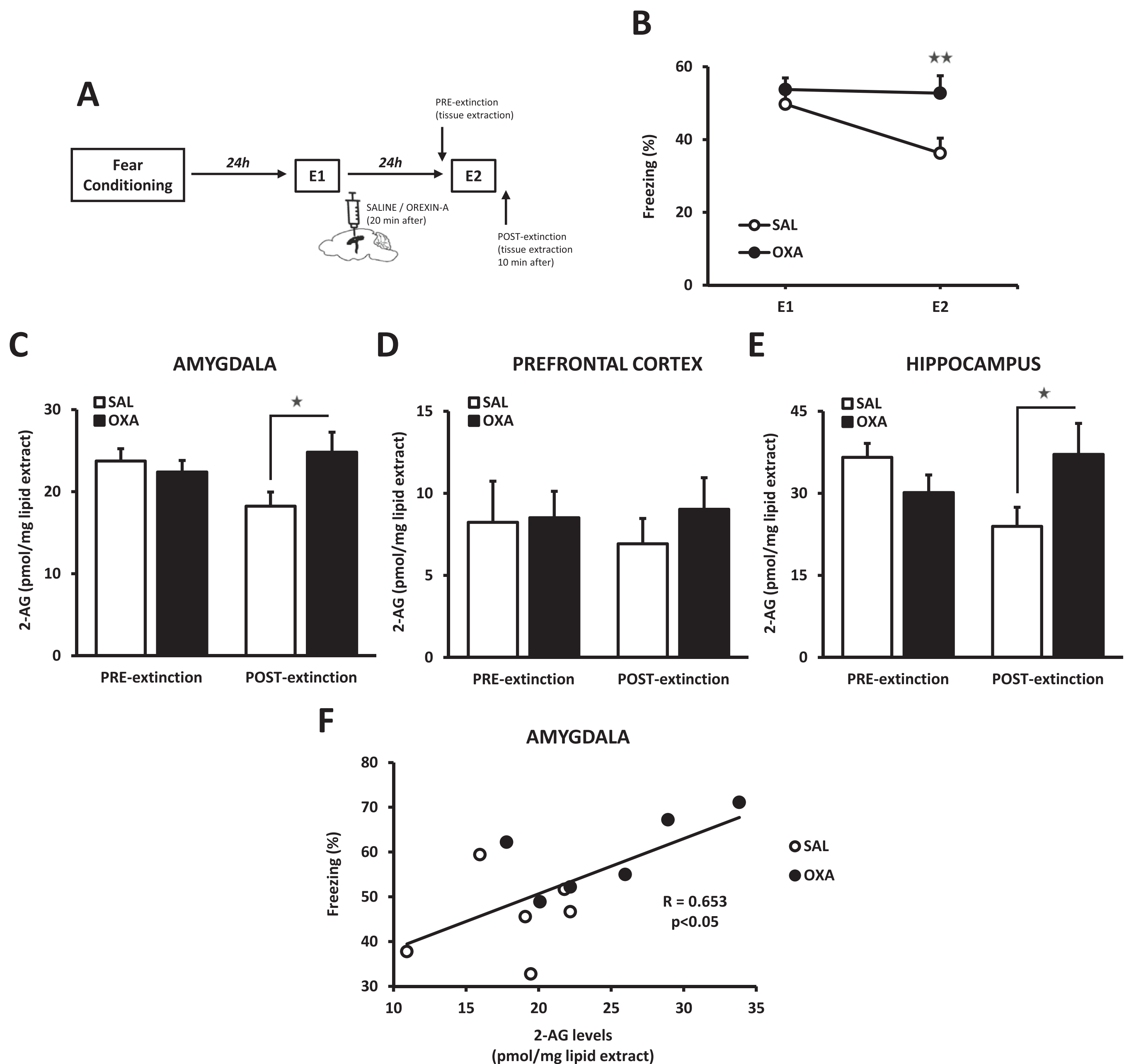

Fig. 2. High levels of 2-AG in the amygdala and hippocampus are associated with impaired fear extinction. (A) Schematic representation of the experimental design for behavioural test. OXA (0.75 nmol·μl^{-1}, icv) or SAL were injected 20 min after E1, and brain tissue was obtained immediately before or 10 min after E2. (B) Time spent freezing by SAL- and OXA-treated mice during E1 and E2 (n = 9–12 mice per group) (interaction day x treatment: $F_{1,19} = 7.69$, $p < 0.05$). (C) 2-AG levels in the amygdala before (pre-extinction) and 10 min after (post-extinction) E2 (interaction experimental condition x treatment: $F_{1,20} = 4.76$, $p < 0.05$) (n = 6 mice per group). (D) 2-AG levels in the prefrontal cortex before (pre-extinction) and 10 min after (post-extinction) E2 (n = 6 mice per group). (E) 2-AG levels in the hippocampus before (pre-extinction) and 10 min after (post-extinction) E2 (interaction experimental condition x treatment: $F_{1,18} = 7.32$, $p < 0.05$) (n = 4–6 mice per group). (F) Correlation between fear memory (freezing values) and 2-AG levels in the amygdala after E2 in mice treated with SAL or OXA 20 min following E1. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ (compared with SAL). OXA: orexin-A; SAL: saline; E1-E2: extinction trials 1–2; 2-AG: 2-arachidonoylglycerol.

tissue was perfused 30 min after the second extinction session (Fig. 3D). Additional control mice were injected with saline or OXA and perfused 24 h later without exposing to footshock (Fig. 3D). In agreement with quantitative RT-PCR analysis, an enhancement of eGFP signal in the BLA (~29%) was found in mice infused with OXA 20 min after the first extinction session in comparison with saline-treated mice (Fig. 3E,J), suggesting an association between impaired extinction and increased eGFP (CB2R) expression. Interestingly, most of the eGFP+ cells (~80%) co-localized with Iba1, a commonly used marker of microglia (Fig. 3H, J), indicating that CB2R-dependent eGFP expression takes place mainly in microglial cells. Moreover, a significant increase in the perimeter of microglia soma was observed in eGFP+ cells (Fig. 3I), independently of saline or OXA treatment. This result indicates that the expression of CB2R is associated with a shift of microglia morphology to a reactive state which is characterized by larger amoeboid soma [34]. No changes in eGFP signal were observed either in the prefrontal cortex or the hippocampus due to OXA infusion (Fig. 3F,G). Basal expression of CB2R was scarce as shown by the low eGFP immunoreactivity in mice that were not exposed to footshock, in the different brain areas evaluated (Fig. 3E,F,G). Moreover, OXA administration by itself did not modify CB2R expression (Fig. 3E,F,G). Taken together, these data suggest that fear extinction deficits induced by OXA are associated with increased

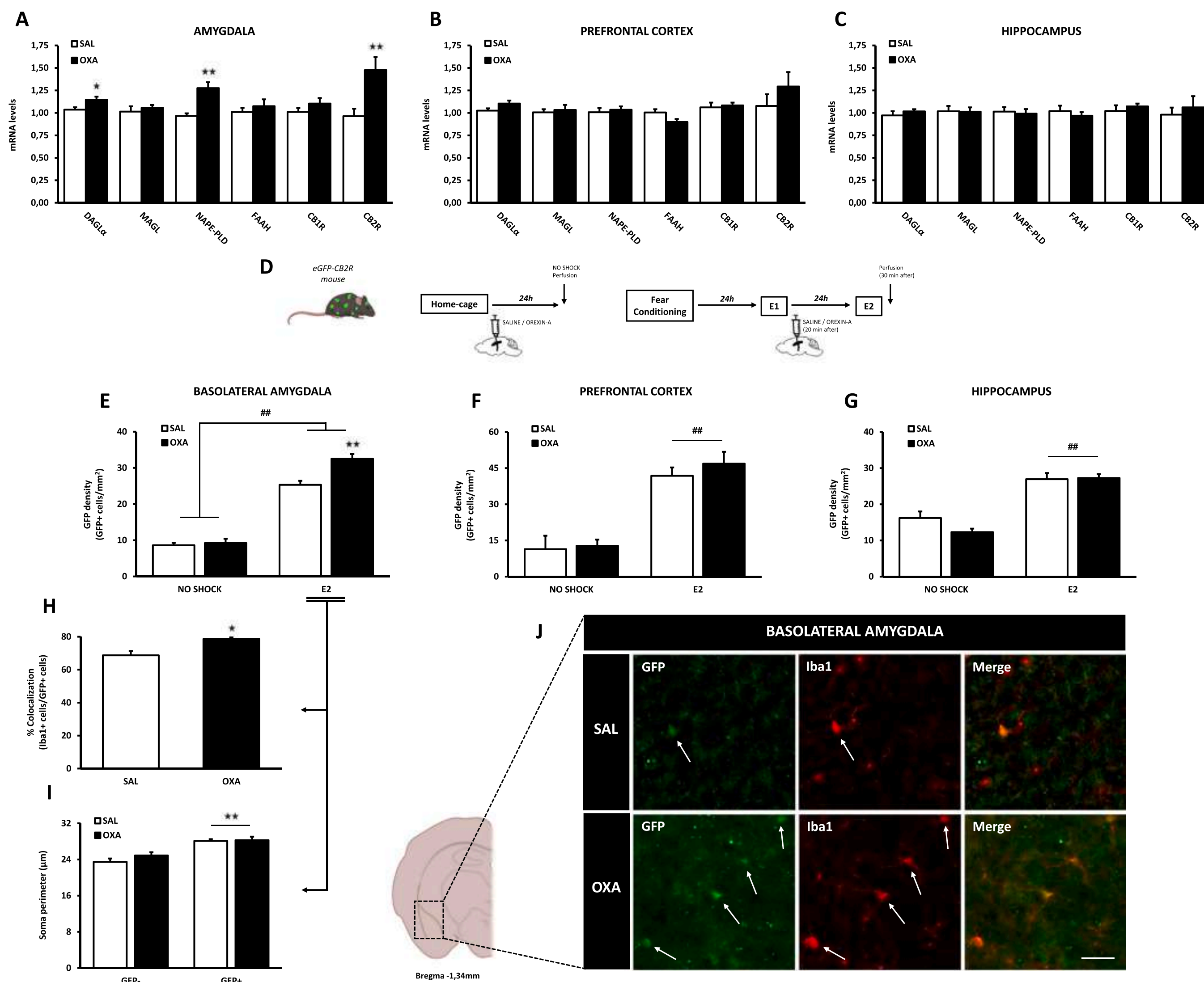

Fig. 3. Impaired fear extinction induced by OXA is associated with increased expression of CB2R in the amygdala. (A,B,C) Gene expression of the endocannabinoid synthesizing and degrading enzymes, CB1R and CB2R in amygdala (A), prefrontal cortex (B) and hippocampus (C) 10 min after E2 in mice treated with SAL or OXA (0.75 nmol·µl, icv) 20 min following E1 (n = 8–12 mice per group). (D) Schematic representation of the experimental design for (E-I) biochemical experiments. (E,F, G) GFP staining in the basolateral amygdala (interaction experimental condition x treatment: $F_{1,11} = 7.59$, $p < 0.05$) (E), prefrontal cortex (experimental condition: $F_{1,10} = 43.89$, $p < 0.001$) (F) and hippocampus (experimental condition: $F_{1,10} = 76.28$, $p < 0.001$ (G) of eGFP-CB2R mice injected with SAL or OXA and sacrificed 24 h later without receiving footshock, and mice exposed to fear conditioning and sacrificed 30 min after E2, treated with SAL or OXA 20 min following E1 (n = 4–5 mice per group). (H) Percentage of GFP+ cells expressing Iba1 in the basolateral amygdala of eGFP-CB2R mice after E2, treated with SAL or OXA 20 min following E1 (n = 4–5 mice per group). (I) Soma perimeter of microglia in GFP+ and GFP- cells in the basolateral amygdala of eGFP-CB2R mice sacrificed after E2, treated with SAL or OXA 20 min following E1 (eGFP effect: $F_{1,14} = 37.35$, $p < 0.01$) (n = 4–5 mice per group). (J) Representative images of the basolateral amygdala of eGFP-CB2R mice labelling GFP (green), Iba1 (red) and colocalization of GFP and Iba1. The sale bar represents 20 µm. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 (compared with SAL or GFP- cells); ##p < 0.01 (compared with NO SHOCK group). OXA: orexin-A; SAL: saline; E1-E2: extinction trials 1–2.

expression of CB2R in microglial cells of the BLA.

3.4. CB2R mediates the impairment of fear extinction induced by OXA

2-AG is a full agonist of CB1R and CB2R [35]. Therefore, in view of the role played by 2-AG in the impairment of fear extinction induced by OXA, we studied the cannabinoid receptor subtype involved in this effect. A low dose of the CB1R antagonist rimonabant (0.5 mg·kg^{-1}, ip) (Fig. 4A) was used to rule out an intrinsic effect of this cannabinoid ligand. Interestingly, pretreatment with rimonabant potentiated the resistance of fear extinction induced by OXA (0.75nmol·µl^{-1}, icv) (Fig. 4B,C). On the contrary, blockade of the CB2R with the specific antagonist AM630 (3 mg·kg^{-1}, ip) (Fig. 4D) completely prevented the extinction deficit induced by OXA (Fig. 4E,F). Locomotor activity was not modified in mice 24 h after acute administration of OXA (0.75nmol·µl^{-1}, icv), rimonabant (0.5 mg·kg^{-1}, ip), and AM630 (3 mg·kg^{-1}, ip) (Fig. S7). Pretreatment with rimonabant or AM630 before OXA also did not alter locomotion 24 h later (Fig. S7). Moreover, rimonabant injection (0.5 mg·kg-1, ip) before OXA (0.75nmol·µl^{-1}, icv) during four days did not induce changes in locomotor activity measured 24 h after the last administration day (fifth day) (Fig. S8). These data demonstrate that the changes observed in freezing behaviour were not due to unspecific effects on locomotion. As a whole, these results are in agreement with the increased expression of CB2R previously observed in the BLA of mice treated with OXA. Moreover, the potentiation of the extinction deficits induced by the per se inactive dose of rimonabant could reflect a blockade of a compensatory and/or simultaneous facilitation of fear extinction through activation of CB1Rs.

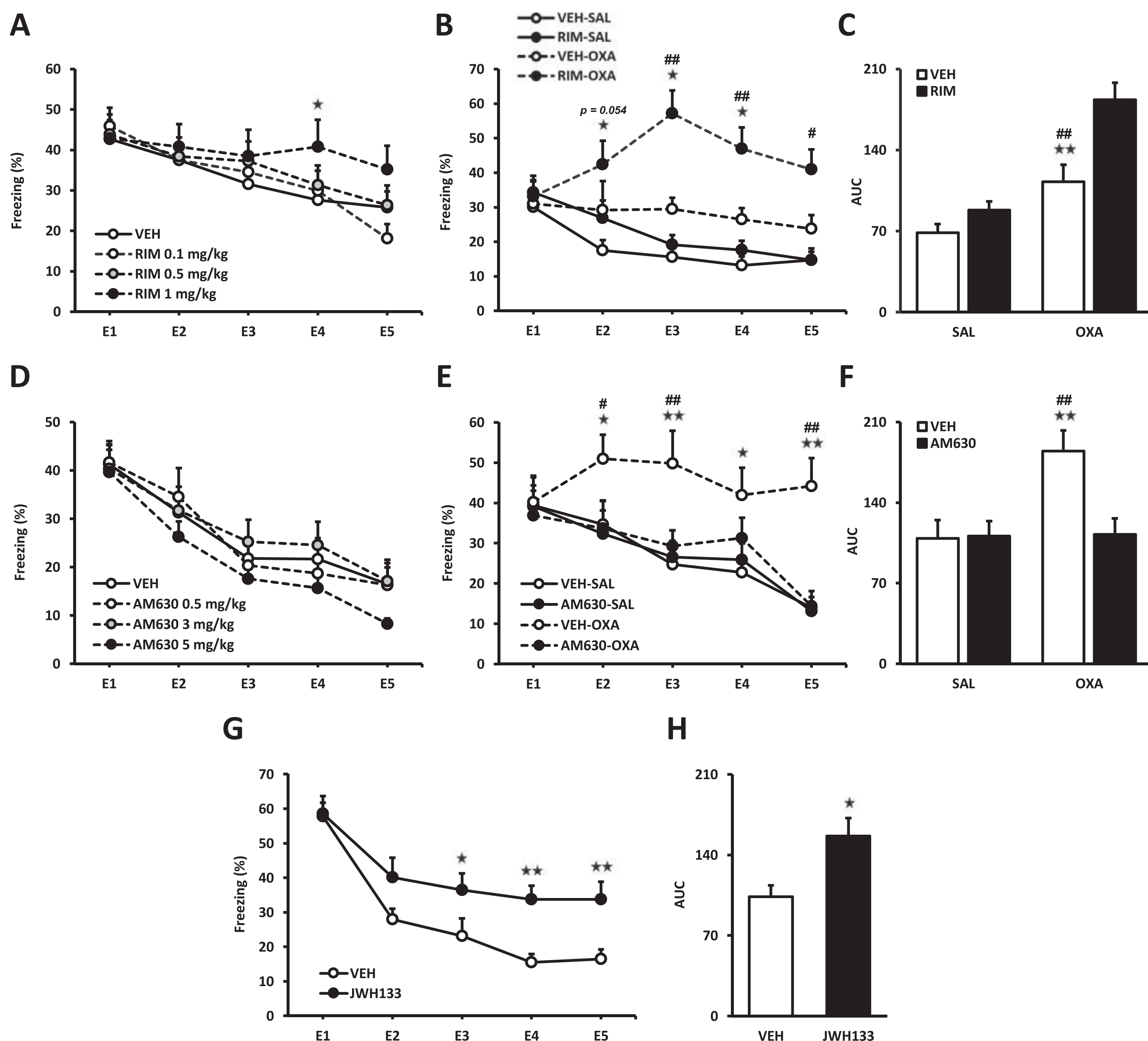

Fig. 4. Impaired fear extinction induced by OXA is mediated by CB2R. (A,D) Time course of the freezing levels during contextual extinction trials in mice treated with the CB1R antagonist rimonabant (0.1, 0.5 and 1 mg·kg^{-1}, ip) (A) or the CB2R antagonist AM630 (0.5, 3 and 5 mg·kg^{-1}, ip) (D) immediately after each extinction session (n = 12–24 mice per group). (B,C,E,F) Time course of the freezing levels during contextual extinction trials (interaction pretreatment x treatment: $F_{1,35}$ = 4.53, $p < 0.05$) (B), (interaction pretreatment x treatment: $F_{1,38}$ = 5.80, $p < 0.05$) (E) and AUC values (interaction pretreatment x treatment: $F_{1,35}$ = 5.28, $p < 0.05$) (C), (interaction pretreatment x treatment: $F_{1,38}$ = 5.78, $p < 0.05$) (F) in mice treated with rimonabant (0.5 mg·kg^{-1}, ip) (n = 7–12 mice per group) (B,C) or AM630 (3 mg·kg^{-1}, ip) (n = 9–11 mice per group) (E,F) immediately after each extinction session, 20 min before OXA (0.75 nmol·µl^{-1}, icv) infusion. (G,H) Time course of the freezing levels during contextual extinction trials (day x treatment interaction: $F_{4,88}$ = 2.27, $p < 0.05$) (G) and AUC values (H) in mice treated with the CB2R agonist JWH133 (2 mg·kg^{-1}, ip) immediately after each extinction session (n = ^{1}1–13 mice per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 (compared with VEH or SAL); #p < 0.05, ##p < 0.01 (comparison between pretreatments). OXA: orexin-A; VEH: vehicle; SAL: saline; E1-E5: extinction trials 1–5; AUC: area under the curve.

Given that CB2Rs underpin the fear extinction deficits of OXA, as suggested by their blockade by AM630, direct-acting CB2R agonists should also promote fear extinction resistance. Accordingly, the CB2R agonist JWH133 (2 mg·kg^{-1}, ip), administered immediately after each extinction session, impaired fear extinction (Fig. 4G,H), confirming a novel functional role for CB2R in the modulation of fear extinction.

3.5. *Intra-amygdala infusion of the CB2R antagonist AM630 prevents the fear extinction deficits promoted by OXA*

Considering the behavioural and biochemical data previously described, we next evaluated the possible direct participation of CB2Rs located in the amygdala in the extinction deficits induced by OXA. For this purpose, mice were bilaterally implanted with cannulae into the BLA and received intra-structure microinjections of AM630 (3 µg/0.5 µl/side) immediately after each contextual extinction session and OXA (0.375nmol/0.5 µl/side) 20 min later. Notably, pretreatment with AM630 into the BLA completely blocked the impaired fear extinction induced by intra-BLA infusion of OXA (Fig. 5A,B). Representative location of the injection sites and a characteristic image of bilateral cannulae positions (Fig. 5C,D) are shown. This result demonstrates an unequivocal role for amygdalar CB2R in the impaired fear extinction

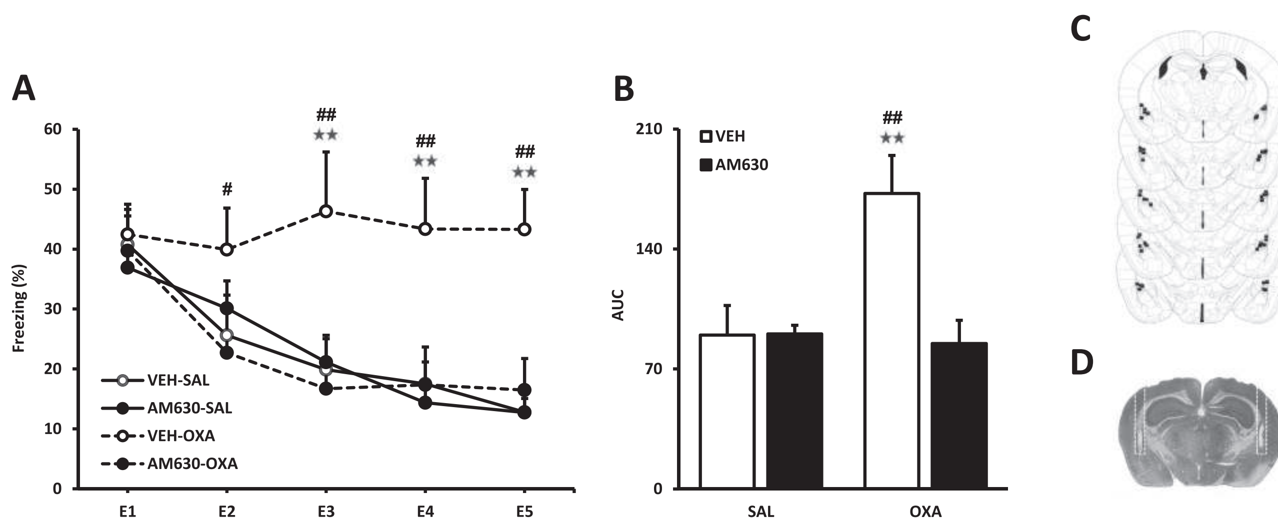

Fig. 5. Intra-amygdala infusion of the CB2R antagonist AM630 blocks the impaired fear extinction induced by intra-amygdala administration of orexin-A. Mice were bilaterally cannulated in the BLA, and after recovery were subjected to contextual fear conditioning and extinction procedure. (A,B) Time course of the freezing levels during contextual extinction trials (interaction pretreatment x treatment: $F_{1,28} = 7.33$, $p < 0.05$) (A), and AUC values (interaction pretreatment x treatment: $F_{1,28} = 7.79$, $p < 0.01$) (B), in mice treated with AM630 (3 μg/0.5 μl/side) immediately after each extinction session, 20 min before OXA (0.375nmol/0.5 μl/side) infusion (n = 8 mice per group). (C) Schematic representation of microinjections sites within the BLA. The number of dots in the figure is fewer than the actual number of animals used because of data overlapping. (D) Photomicrograph of a coronal section of a representative subject showing bilateral injection sites within the mice BLA. Data are expressed as mean ± SEM. **$p < 0.01$ (compared with VEH or SAL); #$p < 0.05$; ##$p < 0.01$ (comparison between pretreatments). OXA: orexin-A; VEH: vehicle; SAL: saline; E1-E5: extinction trials 1–5; AUC: area under the curve.

promoted by OXA.

3.6. Microglial depletion blocks the increase of CB2R expression in the amygdala and prevents the impairment of fear extinction induced by OXA

To determine the contribution of amygdalar CB2R located in microglial cells in the fear extinction deficit induced by OXA, we used PLX5622, a colony-stimulating factor-1 receptor (CSF1R) antagonist, to pharmacologically deplete microglia in animals undergoing the fear extinction process. To this aim, 4 days before starting behavioural evaluation, both saline- and OXA-treated mice were given either control chow or PLX5622 chow (Fig. 5A). Notably, the impairment of fear extinction induced by OXA (0.75nmol·μl^{-1}, icv) was totally prevented in mice exposed to PLX5622 chow (Fig. 5B,C). PLX5622 chow exposure during four days did not modify locomotor activity evaluated 24 h later (Fig. S1). In agreement with previous reports [31,36], microglia were successfully deleted in the amygdala as shown by the dramatic decrease in the expression of the microglial marker CX3CR1 (~80%) by quantitative RT-PCR analysis in saline and OXA-treated mice exposed to PLX5622 chow (Fig. 5D). Moreover, the enhanced expression of CB2R in the amygdala of mice infused with OXA and exposed to control diet that are resistant to fear extinction was markedly decreased (~80%) due to PLX5622 chow exposure (Fig. 5E). However, as previously observed, mRNA expression of CB1R was not affected by either OXA infusion or by PLX5622 chow treatment (Fig. 5F). These data suggest that CB2Rs located in microglial cells of the amygdala may be involved in the fear extinction deficits induced by overactivation of the orexin system. (Fig. 6).

4. Discussion

Our data demonstrate a pivotal role for 2-AG in the impairment of fear extinction induced by OXA. Moreover, we reveal that CB2Rs, specifically those located in the amygdala, are involved in the extinction deficit triggered by OXA.

Orexins are implicated in the modulation of emotional behaviours [3,4], and activation of this system is related to poor extinction of conditioned fear. In humans, patients with panic anxiety symptoms show elevated CSF orexin concentrations [16]. Consistent with this, OX1R blockade facilitated fear extinction in animal models [8,11] and reduced CO2-induced fear and anxiety symptoms in humans [37]. A better understanding of the neurobiological mechanisms by which orexins regulate fear extinction may provide novel pharmacologic targets for PTSD or panic disorders.

OXA triggers biosynthesis of the endocannabinoid 2-AG via the PLC/DAG/DAGLα pathway downstream to OX1R [25], a Gq-protein-coupled receptor. Functional interactions between orexins and endocannabinoids have been reported, mainly in the regulation of pain [38–40], food intake [41,42], and cocaine relapse [43]. A general mechanism of these interactions implies OXA-induced synthesis of 2-AG and subsequent CB1R-dependent retrograde inhibition of GABA release, leading to disinhibition of different pathways in brain areas such as the periaqueductal grey matter [38] or the ventral tegmental area [43].

We investigated the possible involvement of 2-AG in the fear extinction deficit induced by OXA given that the ECS also plays an important role in the modulation of the extinction of aversive memories [20,21]. O7460, a selective inhibitor of DAGLs [44], which are the enzymes in charge of 2-AG biosynthesis, prevented the impaired fear extinction elicited by OXA. Consistently, an early increase of 2-AG, but not AEA, was observed in the amygdala after the infusion of OXA at the same dose that impairs extinction. At a later time point, 2-AG levels increased in the prefrontal cortex, maybe due to an indirect effect of OXA rather than direct orexin-mediated stimulation of 2-AG biosynthesis. Intriguingly, the levels of 2-AG dramatically decreased in the hippocampus at the same later time point. OXA administration could modify, through the alteration of 2-AG levels, the communication between these brain regions that underlies important cognitive and behavioural functions [45]. Indeed, the ECS is a fundamental regulator in synaptic plasticity and functional connectivity in the hippocampus-prefrontal cortex pathway [46].

Our data also demonstrate that high levels of 2-AG are related to resistance of fear extinction. Thus, 2-AG levels increased in the amygdala and the hippocampus, but not in the prefrontal cortex, after the extinction session in mice treated with OXA that did not extinguish fear. A significant correlation between fear memory (freezing values) and 2-AG levels was also found in the amygdala. Moreover, in agreement with

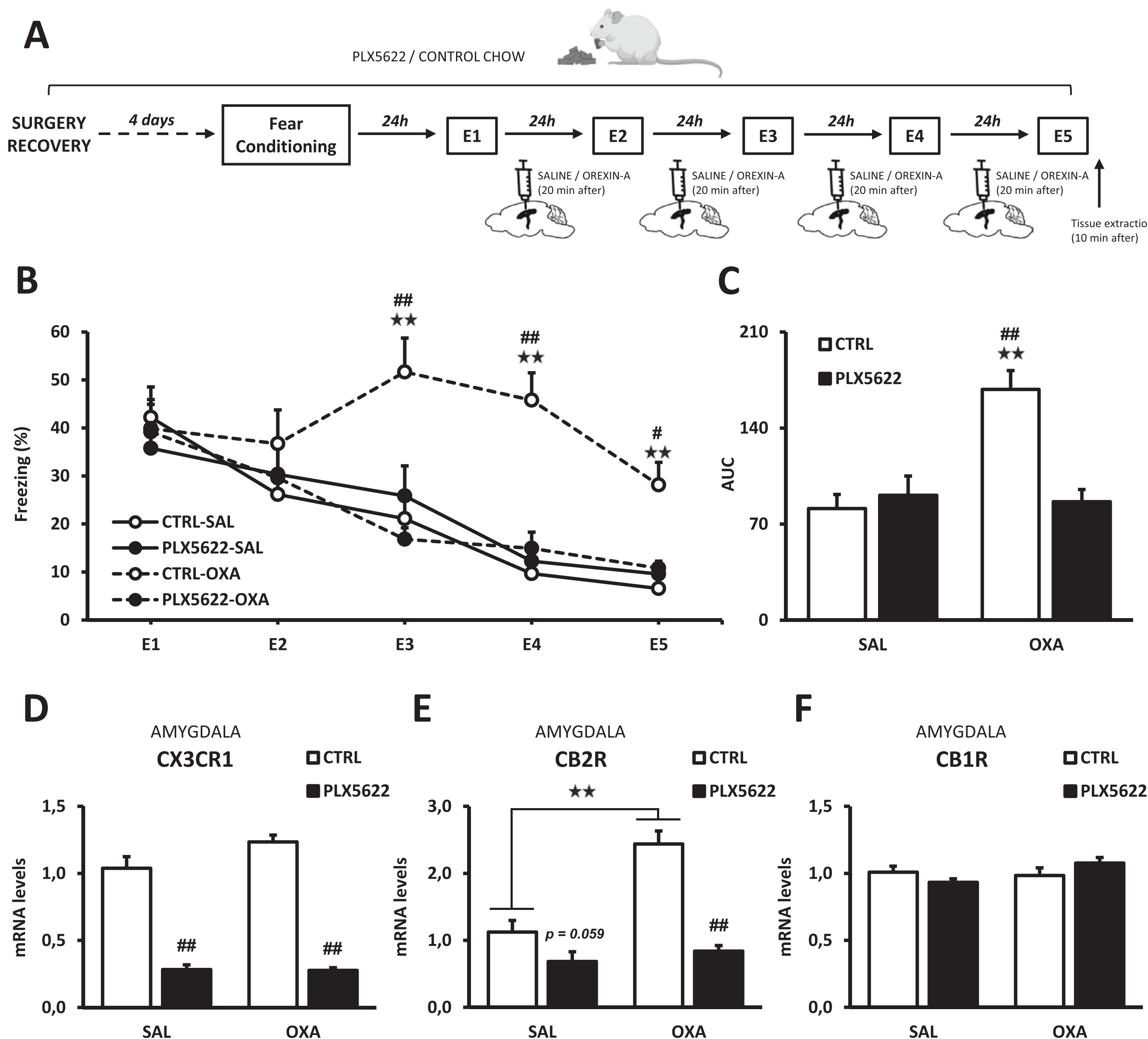

Fig. 6. Microglial and CB2R depletion in the amygdala with PLX5622 chow prevents the impairment of fear extinction induced by OXA. (A) Schematic representation of the experimental design. (B,C) Time course of the freezing levels during contextual extinction trials (interaction day x diet x treatment: $F_{4,152} = 3.78$, $p < 0.01$) (B) and AUC values (interaction diet x treatment: $F_{1,38} = 14.77$, $p < 0.001$) (C) in mice treated with SAL or OXA (0.75 nmol·μl^{-1}, icv) 20 min after each extinction session, and exposed to control or PLX5622 chow (n = 10–11 mice per group). (D-F) Gene expression of CX3CR1 (diet effect: $F_{1,31} = 204.81$, $p < 0.001$) (D), CB2R (interaction diet x treatment: $F_{1,30} = 13.28$, $p < 0.01$) (E), and CB1R (F) in the amygdala of mice treated with SAL or OXA (0.75nmol·μl, icv) 20 min after each extinction session, and exposed to control or PLX5622 chow. Tissue was extracted 10 min after the last extinction session (n = 7–10 mice per group). Data are expressed as mean ± SEM. **p < 0.01 (compared with SAL); #p < 0.05, ##p < 0.01 (comparison between diets). OXA: orexin-A; CTRL: control diet; SAL: saline; E1-E5: extinction trials 1–5; AUC: area under the curve.

previous reports [23], the administration of JZL184, an inhibitor of MAGL which is responsible for 2-AG degradation, impaired fear extinction. Therefore, an optimal level of 2-AG could be required for appropriate processing of fear responses since mice deficient in DAGLα, which have reduced 2-AG brain levels, also exhibit impaired fear extinction [47]. This is feature also of germ-free mice [48], which are also characterised by lower brain 2-AG levels [49]. On the other hand, CB1R knockout mice and wild-type mice treated with the CB1R antagonist rimonabant show resistance to fear extinction [21] and consistently, AEA facilitates fear extinction by CB1R activation in the BLA [22]. Taken together, these data suggest potentially opposing functions of AEA and 2-AG on fear extinction modulation indicating that endocannabinoid signalling could play a more complex role in the regulation of fear learning processes than previously thought.

We next explored the cannabinoid receptor subtype involved in the mediation exerted by 2-AG in OXA-induced impaired fear extinction given that this endocannabinoid, unlike AEA, is a full agonist at CB1R and CB2R [35]. In this study we propose that CB2Rs located in the amygdala are involved in the fear extinction deficit produced by OXA infusion. This proposal is based on the following observations: (i) mRNA levels of CB2R increased in the amygdala, but not in the prefrontal cortex or the hippocampus, in mice treated with OXA and resistant to fear extinction. Importantly, a similar increase of CB2R was observed in the BLA by using eGFP-CB2R mice. Most of CB2Rs colocalized with microglial cells, which were activated by the presence of this cannabinoid receptor subtype; (ii) systemic and intra-BLA CB2R antagonism

with AM630 completely prevented the extinction deficit of OXA; (iii) administration of the selective CB2R agonist, JWH133, induced impairment of fear extinction; (iv) microglia depletion in the amygdala following exposure to PLX5622 chow reduced the increased expression of CB2R in OXA-treated mice, and suppressed the extinction deficit induced by the neuropeptide, suggesting a participation of CB2Rs located in microglial cells of the amygdala in this effect. Conversely, CB1R mRNA was not altered by microglia reduction, consistent with the main localization of this receptor in neurons.

Although the CB2R was initially regarded as a peripheral cannabinoid receptor, several studies indicate that this receptor is expressed in the CNS mainly under pathological conditions. Thus, brain CB2Rs are highly inducible in response to various insults [50–53] and have been involved in the regulation of different neurobiological processes including cognition, and mood-related (anxiety, depression) behaviours [54]. A recent study has shown an anxiolytic-like effect of 2-AG through CB2R activation in a model of innate predator-induced fear [53]. While evidence for a role of CB2R in anxiolytic-like effects is still sparse, MAGL inhibition has emerged as a potential target for anxiolytic drug discovery [55]. However, based on our data and as previously suggested [23], 2-AG signalling and CB2R could play a different role in the modulation of unconditioned anxiety and stress responses versus conditioned fear behaviours, emphasizing the complexity of the ECS involvement in emotional regulation. It is noteworthy that, while deletion of CB2R was found to disrupt the consolidation of foot-shock aversive memories using the step-down inhibitory avoidance test [56], the possible involvement of CB2R in fear extinction learning has not been previously reported. The apparent opposing role for CB1R and CB2R in the regulation of fear extinction could explain why unselective activation of cannabinoid receptors by, e.g., cannabis preparations or Δ^9-tetrahydrocannabinol, has so far been found of controversial efficacy [57,58] to treat these disorders, despite the beneficial role played therein by CB1Rs.

Our data reveal that the impairment of fear extinction induced by the overactivation of the orexin system is mediated by 2-AG and amygdalar CB2R stimulation. Moreover, although the intervention of other cell types cannot be ruled out, our results suggest that microglial cells of the amygdala could be responsible of this effect. A growing body of evidence demonstrates that CB2R is up-regulated in microglia in the context of neuroinflammatory diseases [59]. Given the presence of CB2R in activated microglial cells of the amygdala during the extinction process, future experimental work will be necessary to elucidate the consequences of CB2R-mediated microglial activation during fear extinction. Indeed, altered microglial function and inflammation may contribute to fear dysregulation [60], and has been suggested to underlie the impairment of fear extinction in germ-free or antibiotic-treated mice [48]. Levels of the proinflammatory cytokine TNFα increased in microglia from mice during retention of fear memory [61] while altered blood concentrations of cytokine such as IL6, IL1β or TNFα were associated with PTSD disorder in humans [62].

5. Conclusions

In summary, our multidisciplinary study revealed the involvement of 2-AG and CB2R located in the amygdala in the impaired fear extinction induced by overactivation of the orexin system. In addition, based on our biochemical and behavioural data, microglial CB2Rs in this brain area seem to be involved in this effect, although this statement needs to be confirmed by further research. The discovery of this novel mechanism warrants the study of new approaches in the treatment of disorders characterized by pathological fear.

CRediT authorship contribution statement

Marc Ten-Blanco: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing; **África Flores**: Conceptualization, Formal analysis, Investigation, Methodology, Writing- original draft; **Inmaculada Pereda-Pérez:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft; **Fabiana Piscitelli**: Investigation, Methodology; **Cristina Izquierdo-Luengo**: Formal analysis, Investigation, Methodology; **Luigia Cristino:** Investigation, Methodology; **Julián Romero**: Funding acquisition, Resources; **Cecilia J. Hillard**: Investigation, Resources; **Rafael Maldonado**: Funding acquisition, Supervision, Resources; **Vincenzo Di Marzo**: Conceptualization, Resources, Writing – review & editing; **Fernando Berrendero**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing financial interests or personal relationships with other people or organizations that could inappropriately influence their work in this paper.

Data Availability

All data generated or analysed during this study are available from the corresponding author on reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112925.

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Supplementary Material

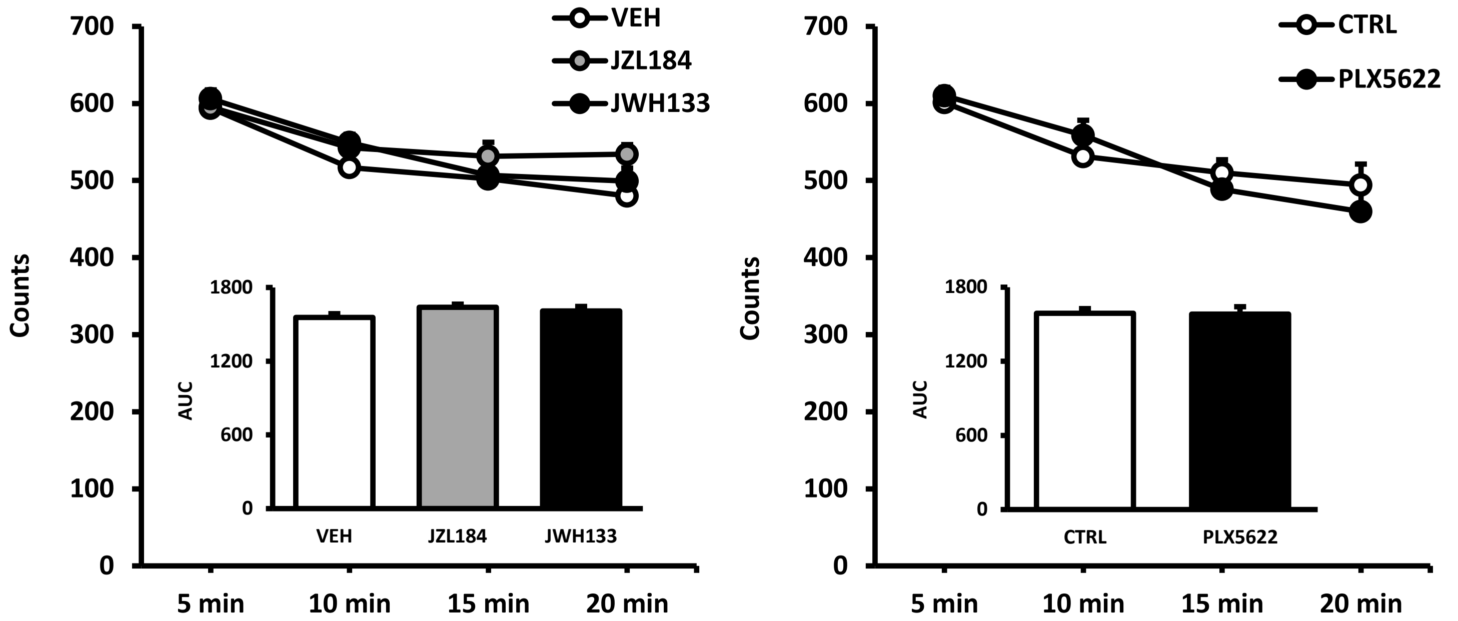

Figure S1. Locomotor activity of VEH-, JZL184 (8 mg·kg^{-1}, ip)- and JWH133 (3 mg·kg^{-1}, ip)-treated mice (*n* = 7-8 mice per group), and mice exposed to CTRL and PLX5622 chow (*n* = 7 mice per group). Locomotion was automatically assessed 24 hours after drug administration every 5 minutes during 20 minutes in locomotor activity cages. Data are expressed as mean ± SEM. AUC, area under the curve; VEH, vehicle; CTRL, control.

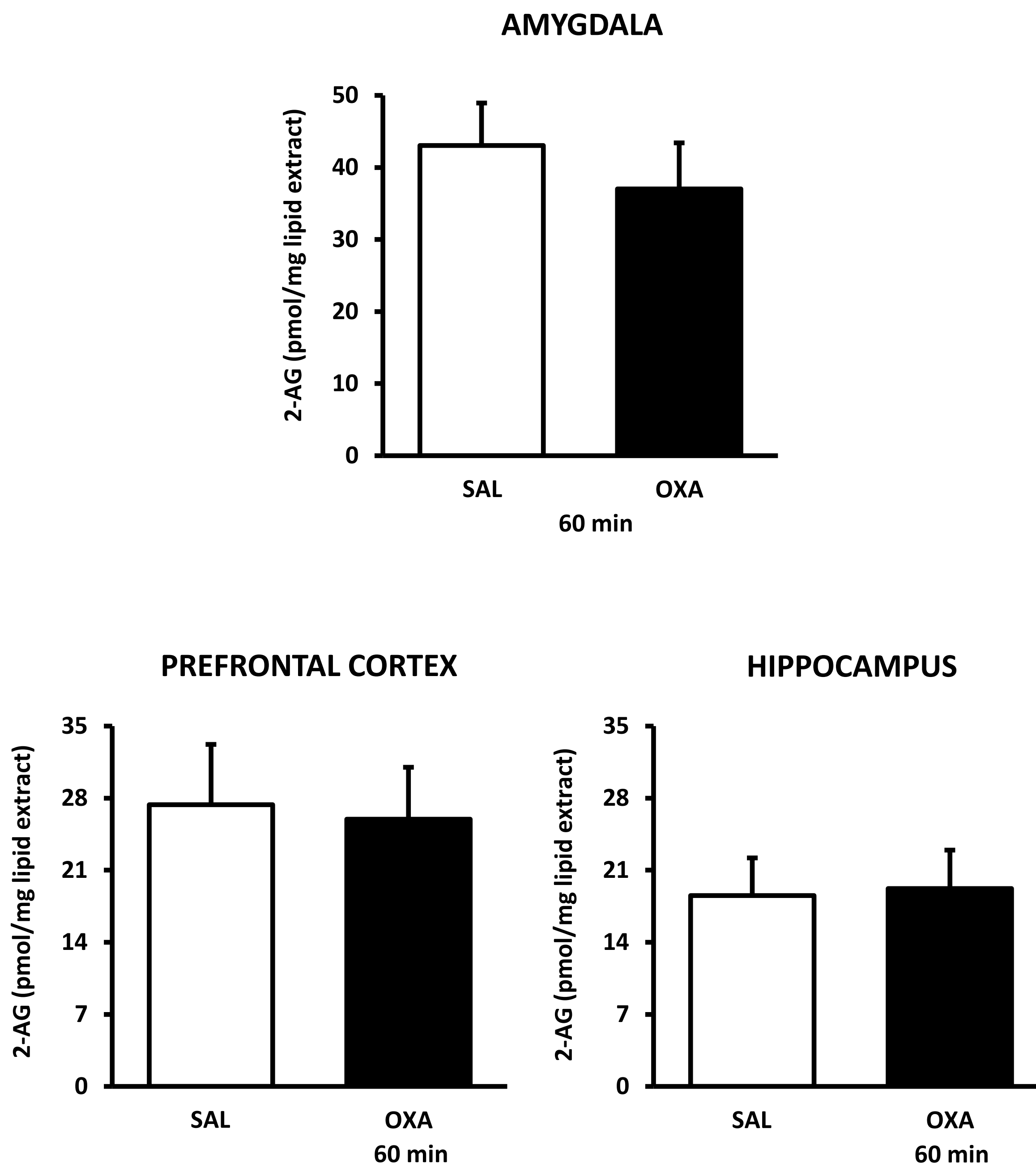

Figure S2. 2-AG levels in amygdala, prefrontal cortex and hippocampus 60 minutes after OXA (0.75 nmol·μl^{-1}, icv) administration (n = 5-6 mice per group). Data are expressed as mean ± SEM. 2-AG, 2-arachidonoylglycerol; SAL, saline; OXA, orexin-A.

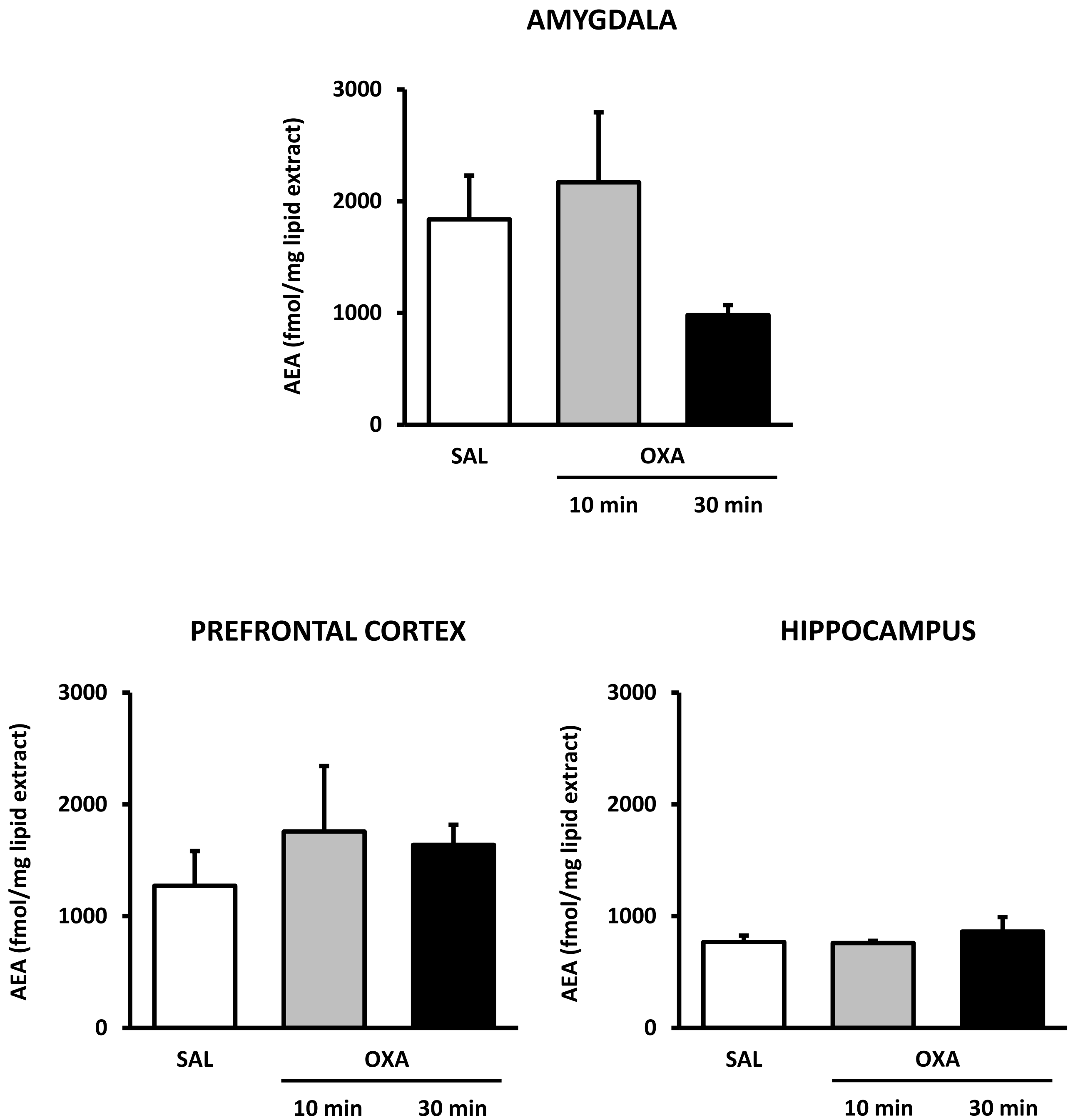

Figure S3. AEA levels in amygdala, prefrontal cortex and hippocampus 10 and 30 minutes after OXA (0.75 $nmol \cdot \mu l^{-1}$, icv) administration (n = 5-6 mice per group). Data are expressed as mean ± SEM. AEA, anandamide; SAL, saline; OXA, orexin-A.

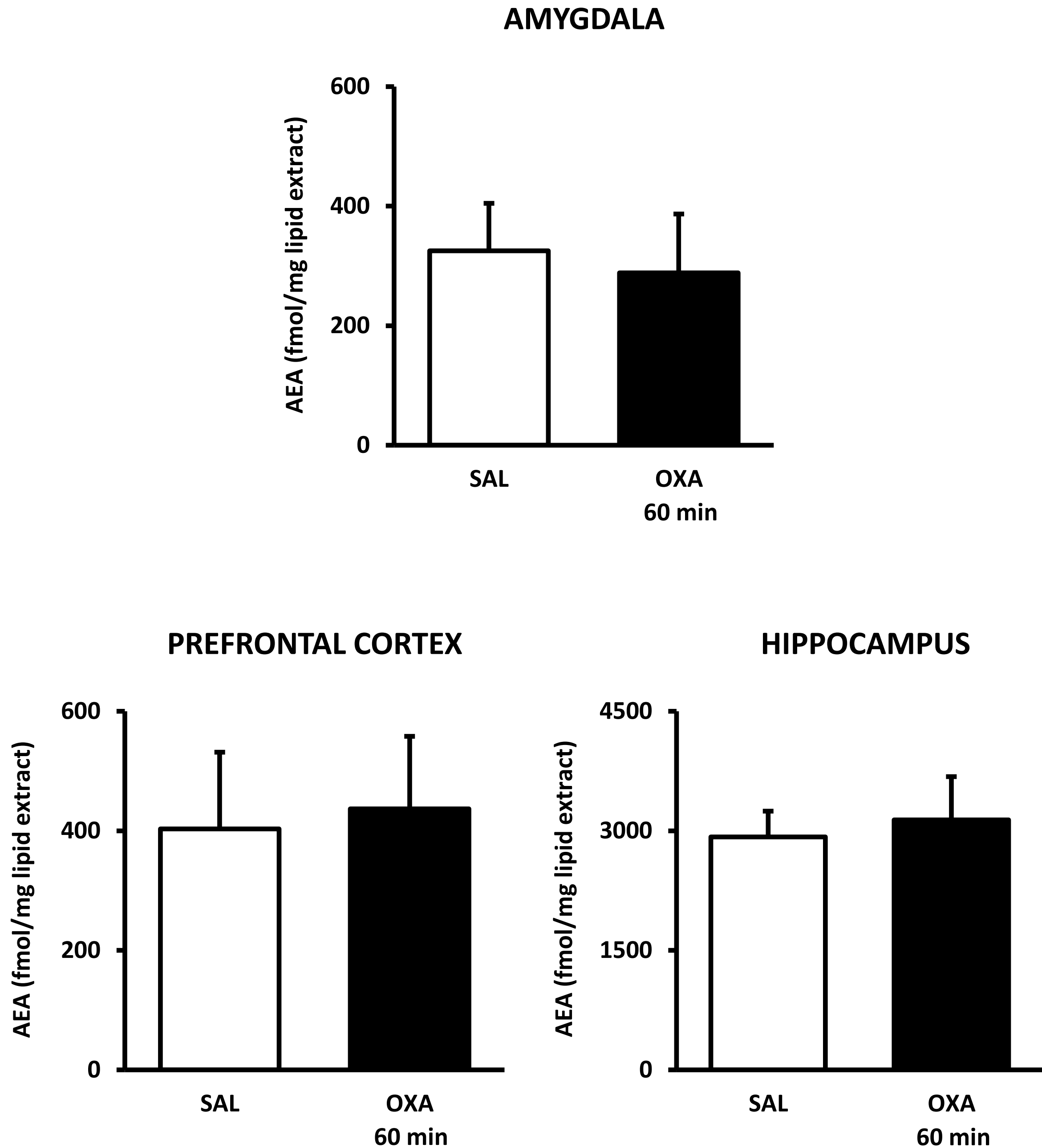

Figure S4. AEA levels in amygdala, prefrontal cortex and hippocampus 60 minutes after OXA (0.75 nmol·μl^{-1}, icv) administration (n = 5-6 mice per group). Data are expressed as mean ± SEM. 2-AG, 2-arachidonoylglycerol; SAL, saline; OXA, orexin-A.

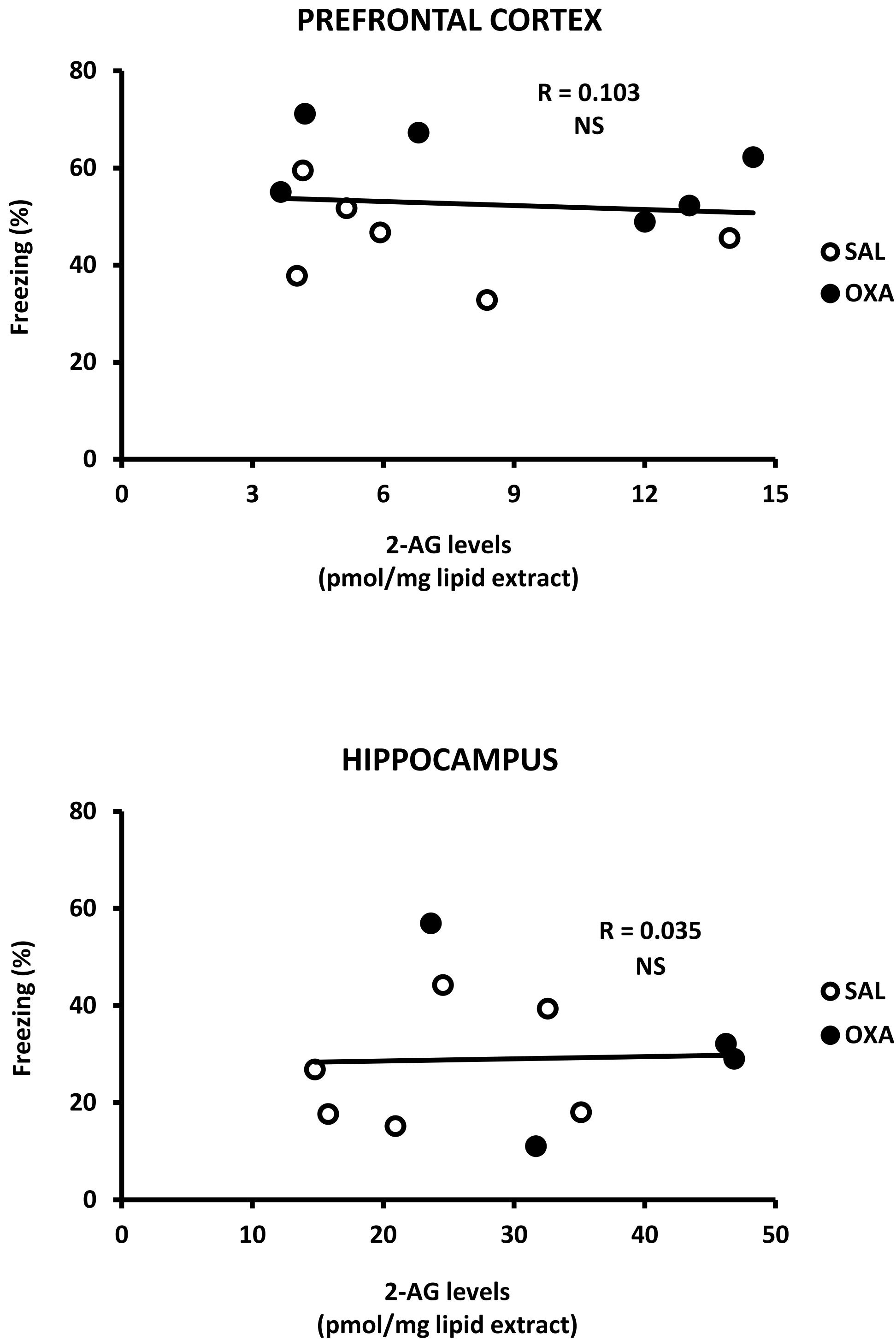

Figure S5. No correlation between 2-AG levels and fear memory (percentage of freezing) in the prefrontal cortex and hippocampus scored during E2 of POST-extinction groups. POST-extinction groups (SAL- and OXA-treated mice (0.75 nmol·µl^{-1}, icv); n = 6 mice per group in the prefrontal cortex; n = 4-6 mice per group in the hippocampus) were sacrificed 10 minutes after E2. OXA, orexin-A; SAL, saline; 2-AG, 2-arachidonoylglycerol; E2, extinction trial 2; NS, non-significance.

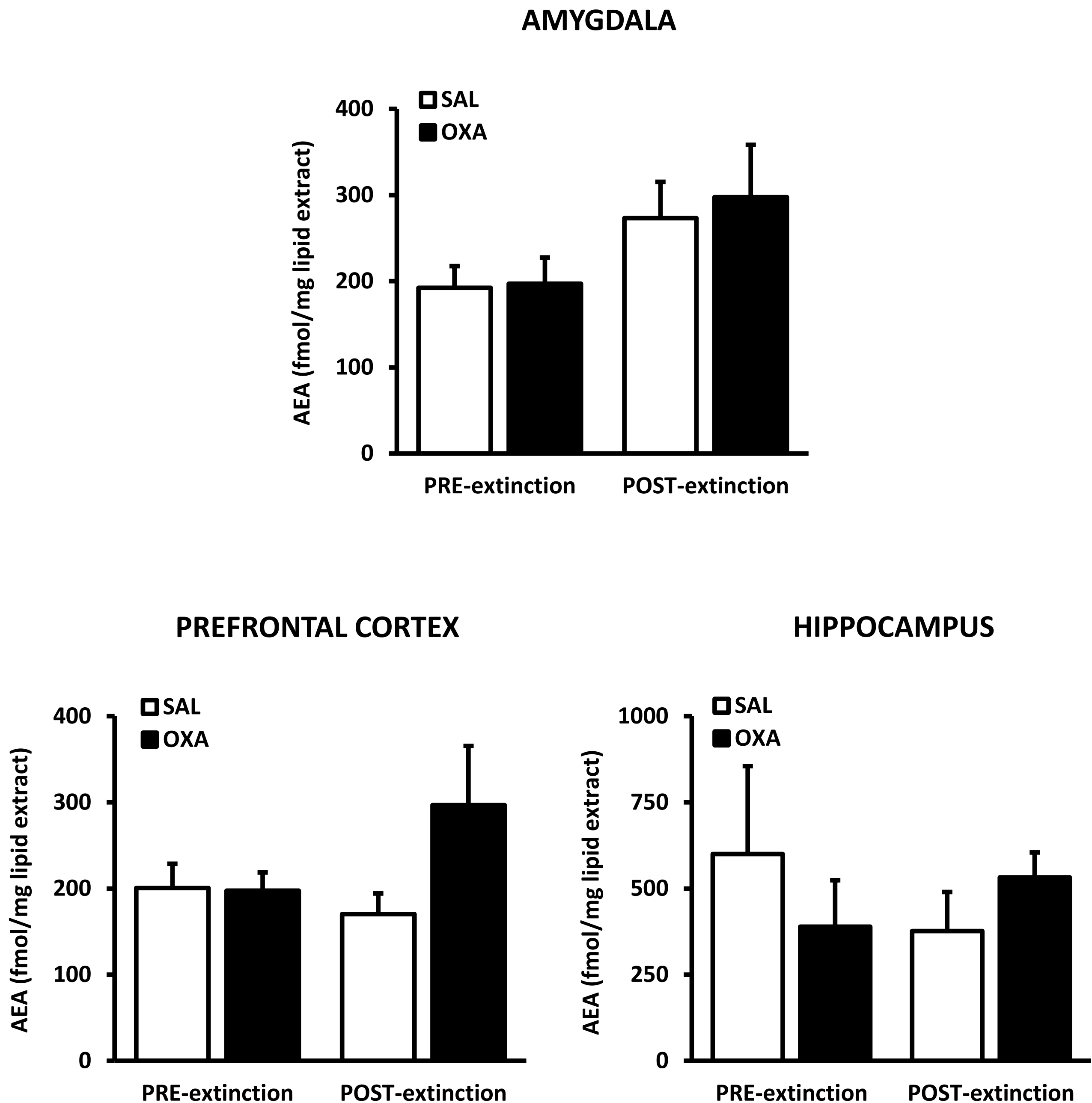

Figure S6. AEA levels in amygdala, prefrontal cortex and hippocampus of SAL- and OXA-treated (0.75 nmol·μl^{-1}, icv) mice in both PRE- and POST-extinction groups. PRE-extinction mice were sacrificed immediately before E2, while POST-extinction mice were sacrificed 10 minutes after E2 (n = 6 mice per group). Data are expressed as mean ± SEM.; AEA, anandamide; SAL, saline; OXA, orexin-A; E1 and E2, extinction trials 1 and 2.

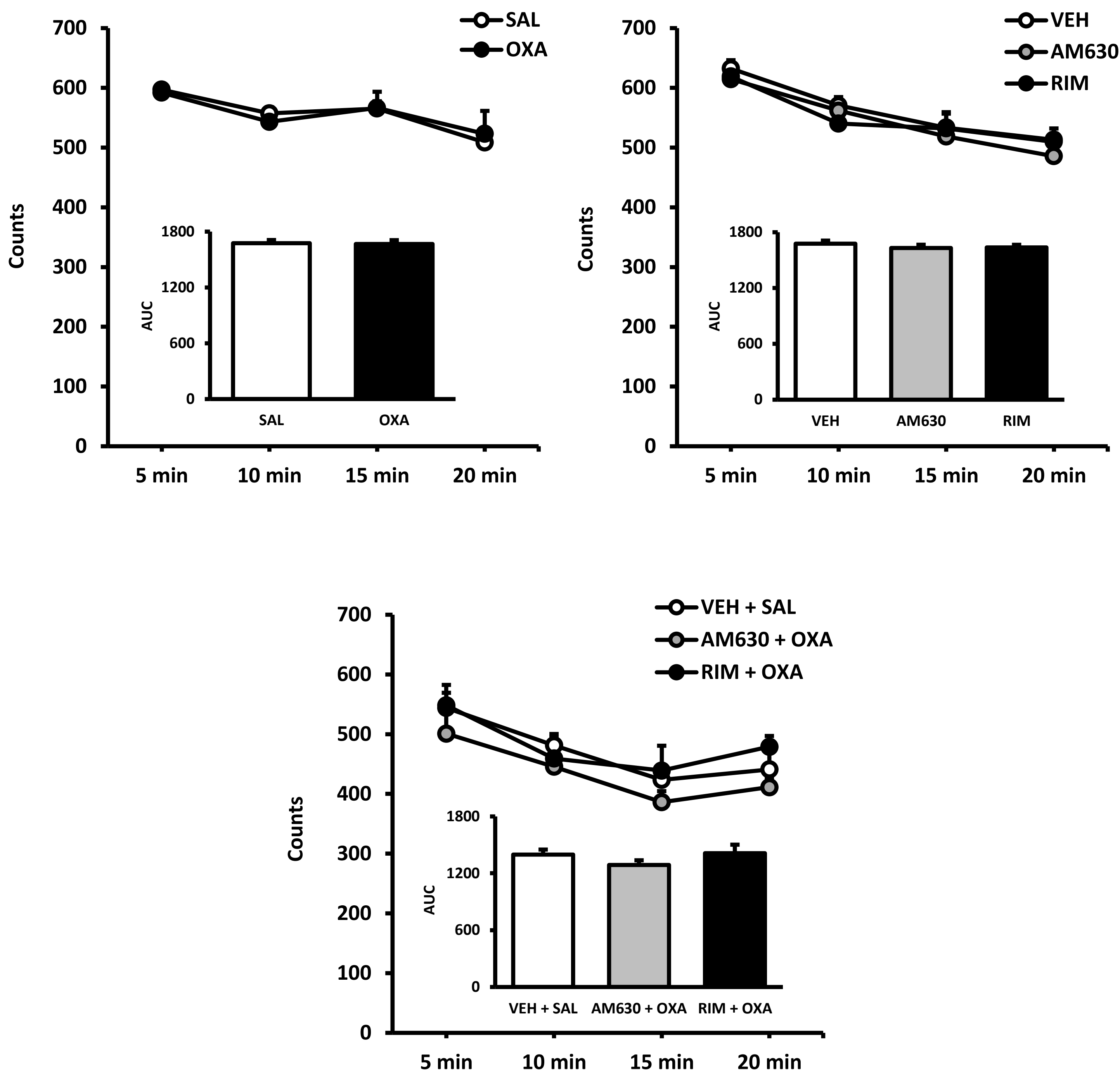

Figure S7. Locomotor activity of SAL- and OXA-treated mice (0.75 nmol·µl^{-1}, icv) (n = 7 mice per group), mice treated with VEH, AM630 (3 mg·kg^{-1}, ip) and rimonabant (0.5 mg·kg^{-1}, ip) (n = 7-8 mice per group), and mice treated with VEH, AM630 (3 mg·kg^{-1}, ip) or rimonabant (0.5 mg·kg^{-1}, ip) 20 minutes before SAL or OXA (0.75 nmol·µl^{-1}, icv) administration (n = 5-6 mice per group). Locomotion was automatically assessed 24 hours after drug administration every 5 minutes during 20 minutes in locomotor activity cages. Data are expressed as mean ± SEM. AUC, area under the curve; SAL, saline; OXA, orexin-A; VEH, vehicle; RIM, rimonabant.

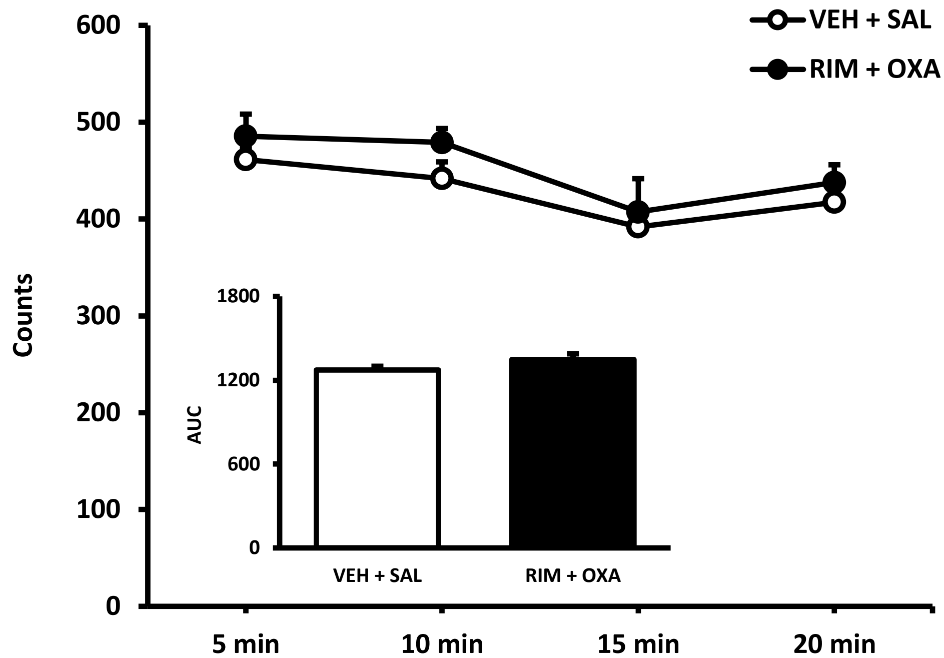

Figure S8. Locomotor activity of mice treated with VEH or rimonabant (0.5 mg·kg^{-1}, ip) 20 minutes before SAL or OXA (0.75 nmol·µl^{-1}, icv) administration during 4 days (n = 6 mice per group). Locomotion was automatically assessed 24 hours after the last drug administration every 5 minutes during 20 minutes in locomotor activity cages. Data are expressed as mean ± SEM. AUC, area under the curve; SAL, saline; OXA, orexin-A; VEH, vehicle; RIM, rimonabant.

Article 3

CB2 cannabinoid receptor expression is increased in 129S1/SvImJ mice: behavioral consequences

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MAIN CONCLUSIONS OF THE ARTICLE

In this article, we observed phenotypic alterations in anxiety, fear extinction and sensorimotor gating in the S1 mouse strain, in comparison to BL6 mice. These changes were associated with a dysregulation of the endocannabinoid system in diverse brain regions, thus highlighting a CB2R increased expression in the amygdala, prefrontal cortex and hippocampus. Then, we demonstrated the involvement of such cannabinoid receptor subtype in the three aforementioned behaviours.

My contributions in the present article are the breeding and maintenance of S1 mice, as well as the participations in the experimental design and in all the behavioural and biochemical experiments. I was also involved in data curation, statistical analysis, and writing the original draft and the corresponding manuscript revisions.

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CB2 cannabinoid receptor expression is increased in 129S1/SvImJ mice: behavioral consequences

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Genetic and environmental factors are implicated in the etiology of neuropsychiatric diseases. Inbred mouse strains, including the 129S1/SvImJ (S1), constitute important models to study the influence of genetic factors in these conditions. S1 mice displayed anxiogenic-like behavior, impaired fear extinction, and increased prepulse inhibition (PPI) of startle reflex compared to C57BL/6J (BL6) mice. Given the role played by the endocannabinoid system (ECS) in these responses, we evaluated the expression of the ECS components in different brain regions in S1 mice. Gene expression levels of the cannabinoid type-1 and type-2 receptors (CB1R and CB2R) and the endocannabinoid metabolizing enzymes varied depending on the brain region evaluated. Notably, CB2R expression markedly increased in the amygdala, prefrontal cortex and hippocampus in S1 mice. Moreover, CB2R blockade with SR144528 partially rescued the anxiogenic phenotype in S1 mice, while CB2R activation with JWH133 potentiated the deficits in fear extinction and the PPI of startle reflex in this mouse strain. These data suggest that CB2R is involved in the behavioral alterations observed in S1 mice and underline the importance of this cannabinoid receptor subtype in the regulation of certain central nervous system disorders.

KEYWORDS

anxiety, fear, prepulse inhibition, CB2 cannabinoid receptor, mouse

Introduction

Genetic predisposition and environmental factors contribute to the development of psychiatric disorders (Uher, 2014). However, clearly more research is needed to fully understand the causes underlying individual differences in risk and resilience for these diseases, including genetic variation. Diverse genetically inbred mouse strains exist, which represent exceptional models for studying the influence of genetic factors in neuropsychiatric disorders (Moore et al., 2020). In this sense, the inbred 129S1/SvImJ (S1) mouse strain displays poor fear extinction (Hefner et al., 2008), dysregulated hypothalamic-pituitary-adrenal axis function (Camp et al., 2012), behavioral alterations associated with increased stress reactivity (Rodriguez et al., 2020), and

sleep disturbances (Fritz et al., 2021). Therefore, this strain may represent a useful model to elucidate distinct and overlapping mechanisms underlying different maladaptive behaviors.

Considering the range of possible neurobiological mechanisms involved in the S1 mice phenotype, the endocannabinoid system (ECS), composed of two main receptors, the cannabinoid type-1 and type-2 receptors (CB1R and CB2R, respectively), their ligands, i.e., the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes involved in endocannabinoid metabolism (Mechoulam and Parker, 2013) could be a promising candidate. This neuromodulatory system plays a crucial role in different neurophysiological processes. Disturbances in the ECS, mainly related to CB1R dysfunction, are associated with several psychiatric conditions such as posttraumatic stress disorder (Mayo et al., 2022), anxiety (Petrie et al., 2021) or schizophrenia (Leweke et al., 2018), among others. Interestingly, CB2R, initially regarded as a peripheral cannabinoid receptor, has been recently involved in the regulation of different neurobiological processes including cognition and mood-related (anxiety, depression) behaviors (Banaszkiewicz et al., 2020). In agreement with potential modifications of the ECS as a molecular mechanism contributing to the phenotypic alterations observed in S1 strain, the selective fatty acid amide hydrolase (FAAH) inhibitor AM3506 rescued fear extinction deficits in these mice (Gunduz-Cinar et al., 2013) by increasing AEA levels in the amygdala. This effect was dependent on CB1R activation in this brain region since the fear-reducing effects of systemic AM3506 were blocked by intra-amygdala infusion of the CB1R antagonist rimonabant (Gunduz-Cinar et al., 2013).

The aim of this study was to analyze the expression of the main components of the ECS in several brain areas of S1 strain compared to C57BL/6J (BL6) mice. Considering the main change observed, we also evaluated the consequences of the modulation of CB2R in S1 mice in key neurobehavioral responses such as anxiety, fear conditioning and extinction, and sensorimotor gating.

Material and methods

Animals

Experiments were performed using male 129S1/SvImJ (S1) mice (Jackson Laboratories) and C57BL/6J (BL6) mice (Charles River) at 8–10 weeks old. BL6 mice were chosen as the comparison strain in this study because they represent one of the most commonly used mouse lines in neuroscience research. Moreover, previous work evaluating fear extinction in the S1 strain typically used BL6 mice as a reference, since they exhibit proper fear extinction, acquisition and recall (Rodriguez et al., 2020). Mice were housed by strain (maximum 5 per cage) and maintained in a temperature (21.1 ± 1°C)- and humidity (55% ± 10%)-controlled room under a 12-h light/dark cycle (lights on at 8:00 a.m.). Food and water were available *ad libitum*. All experiments were performed during the light phase. Mice were handled daily for 3 days before the beginning of the experiment. Experimental procedures were conducted in the animal facilities of Universidad Francisco de Vitoria in Madrid, Spain, in accordance with the guidelines of the European Communities Directive 2010/63/EU and the Spanish Regulations RD 1201/2005 and 53/2013 regulating animal research and approved by the local ethical committee (CEEA-UFV).

Drugs

The CB2R agonist JWH133 (5 mg/kg) (Tocris) was dissolved in a solution of 10% DMSO, 10% Tween 80 and 80% saline. The CB2R antagonist SR144528 (3 mg/kg) (Sigma) was dissolved in a solution of 5% ethanol, 5% cremophor and 90% saline. Both drugs were administered by intraperitoneal (ip) route (10 ml/kg body weight). Doses were based on previous studies in mice (Busquets-Garcia et al., 2011; Donvito et al., 2017) in mice.

Elevated plus maze test

Anxiety-like behavior was assessed by using a black maze elevated 30 cm above the ground with four arms (25 cm × 5 cm) set in a cross from a neutral central square (5 cm × 5 cm). Two opposite arms were delimited by walls (closed arms) and illuminated with 4–6 lux, whereas the two other opposite arms had unprotected edges (open arms) and were illuminated with 40–50 lux. Pharmacological treatments were administered 30 min before the test. The total number of visits to the closed and open arms, and the cumulative time spent in each arm were observed through a videocamera system during 5 min.

Cued fear conditioning and extinction

Mice were cued fear-conditioned as performed in preceding experiments with slight modifications (Flores et al., 2014). The test chamber (LE116, Panlab, Harvard Instruments) was made with black methacrylate walls and a transparent front door. This chamber (25 cm × 25 cm × 25 cm) was located inside a soundproof module with a ventilation fan in order to provide a background noise and attenuate surrounding sounds. The chamber floor was constructed of parallel stainless-steel bars of 2 mm of diameter spaced at 6 mm intervals and was connected to a scrambled shock generator (LE100-26 module, Panlab, Harvard Instruments). A high-sensitivity weight transducer (load cell unit) was used to record and analyze the signal generated by the animal movement intensity. Experimental software PACKWIN V2.0 automatically calculated the percentage of

immobility time for each experimental phase. Before each trial, the chamber floor and walls were cleaned with 70% ethanol and then water to avoid olfactory cues. On the conditioning session, mice were individually placed in the chamber during 180 s before the onset of three cue tones (3 kHz, 90 dB, 30 s long, 10 s between tones), each one co-terminating with a footshock (0.7 mA, 1 s). After the last cue tone, mice remained in the chamber for 10 s. Fear extinction sessions (E1–E5) were performed 24, 48, 72, 96 and 120 h after the conditioning day in a different context (transparent Plexiglas cylinder surrounded by white walls and a smooth floor). In E1, mice were habituated to the new context during 180 s, whereas in E2–E5 this habituation time was reduced to 60 s. After the habituation, mice were re-exposed to the CS (4 cue tones, 30 s long, 10 s between tones). To study the fear extinction process, pharmacological treatments were administered 30 min before each extinction session. Fear memory was assessed as the percentage of time that mice spent freezing during the 4 cue tones of each extinction session. Freezing behavior, a rodent's natural response to fear, was automatically evaluated and defined as complete lack of movement, except for breathing for more than 800 ms. Data from fear extinction were expressed as percentage of freezing behavior and as area under the curve (AUC). AUC was calculated by using a standard trapezoid method, $AUC = [0.5 \times (B1 + B2) \times h] + [0.5 \times (B2 + B3) \times h] + \ldots [0.5 \times (Bn + Bn + 1) \times h]$, where Bn were the percentage of freezing behavior for each mouse and h was the time (days) passed between the consecutive measurements.

Prepulse inhibition of startle reflex

Prepulse inhibition (PPI) of startle reflex, a measure of sensorimotor gating, was assessed by using the StartFear Combined System (Panlab, Harvard Instruments). Mice were daily habituated to a Plexiglas cylinder located inside the sound-attenuating chamber for 5 min with background white noise (65 dB) 4 days prior to the test. The test started with 5 min habituation in the cylinder and, immediately after, mice were exposed to 5 pulse trials (120 dB, white noise, 40 ms). These trials were performed for startle accommodation and were excluded in the final analysis. The experimental protocol consisted of 10 blocks with 6 or 12 trials each, randomly presented to mice with an inter-trial interval of 7–23 s. Blocks consisted of: no stimulus (6×) (background white noise), pulse alone (12×) (120 dB, white noise, 40 ms), pulse preceded by 4 prepulse intensities (12× each) (4, 8, 12 and 16 dB above background noise, 20 ms, 100 ms before pulse) and the prepulses alone (6× each). A background white noise was generated throughout the whole experiment. Pharmacological treatments were administered 30 min before the test. Startle amplitude was detected by PACKWIN V2.0 software. Percent PPI was calculated as follows: 100 × (startle response – prepulse inhibited startle response) / startle response.

Quantitative RT-PCR analysis

Amygdala, prefrontal cortex and hippocampus tissues were extracted in basal conditions and immediately frozen at −80°C. These brain areas were chosen based on their implication in the behavioral responses evaluated in this study. Total RNA was purified with the RiboPure™ Kit (Invitrogen) for amygdala and prefrontal cortex, and the RNeasy Mini Kit (QIAGEN) for hippocampus, according to the manufacturer's instructions. Reverse transcription was performed with 0.9 μg of total RNA and the SuperScript™ II Reverse Transcriptase (Invitrogen). PCR reactions were conducted using PrimePCR™ Probe Assay (Bio-Rad) to quantify mRNA levels for: CB1R (Unique Assay ID: qMmuCEP0038879), CB2R (Unique Assay ID: qMmuCEP0039299), DAGLα (Unique Assay ID: qMmuCIP0032590), MAGL (Unique Assay ID: qMmuCIP0042348), NAPE-PLD (Unique Assay ID: qMmuCIP0035707) and FAAH (Unique Assay ID: qMmuCEP0055480), using GAPDH expression (Unique Assay ID: qMmuCEP0039581) as endogenous control gene for normalization. PCR assays were carried out with the CFX Connect Real-Time PCR Detection System (Bio-Rad). The fold changes in gene expression of S1 in comparison with BL6 mice were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Comparisons between two groups were assessed by Student's *t* tests. Multiple-group comparisons were performed by one-way analysis of variance (ANOVA). Repeated-measurement ANOVA was used for serial freezing responses and startle amplitude response between the different prepulse intensities. Subsequent Fisher's LSD post-hoc test was only used when ANOVA interaction effects were significant. All data were expressed as mean ± SEM. The statistical analysis was performed using Statistica (StatSoft) software. The level of significance was $p < 0.05$ in all experiments.

Results

Anxiogenic-like behavior, impaired fear extinction, and increased prepulse inhibition of startle reflex in S1 mice

First, we carried out a direct comparison between S1 and BL6 mice in several neurobehavioral responses. Unconditioned anxiety was evaluated by using the elevated plus maze (EPM). S1 mice showed an anxiogenic-like effect ($p < 0.01$) (Figure 1A) as revealed the decrease of the percentage of time spent in open arms. No changes were observed in the total number of entries (Figure 1B). Cued fear conditioning was not modified in

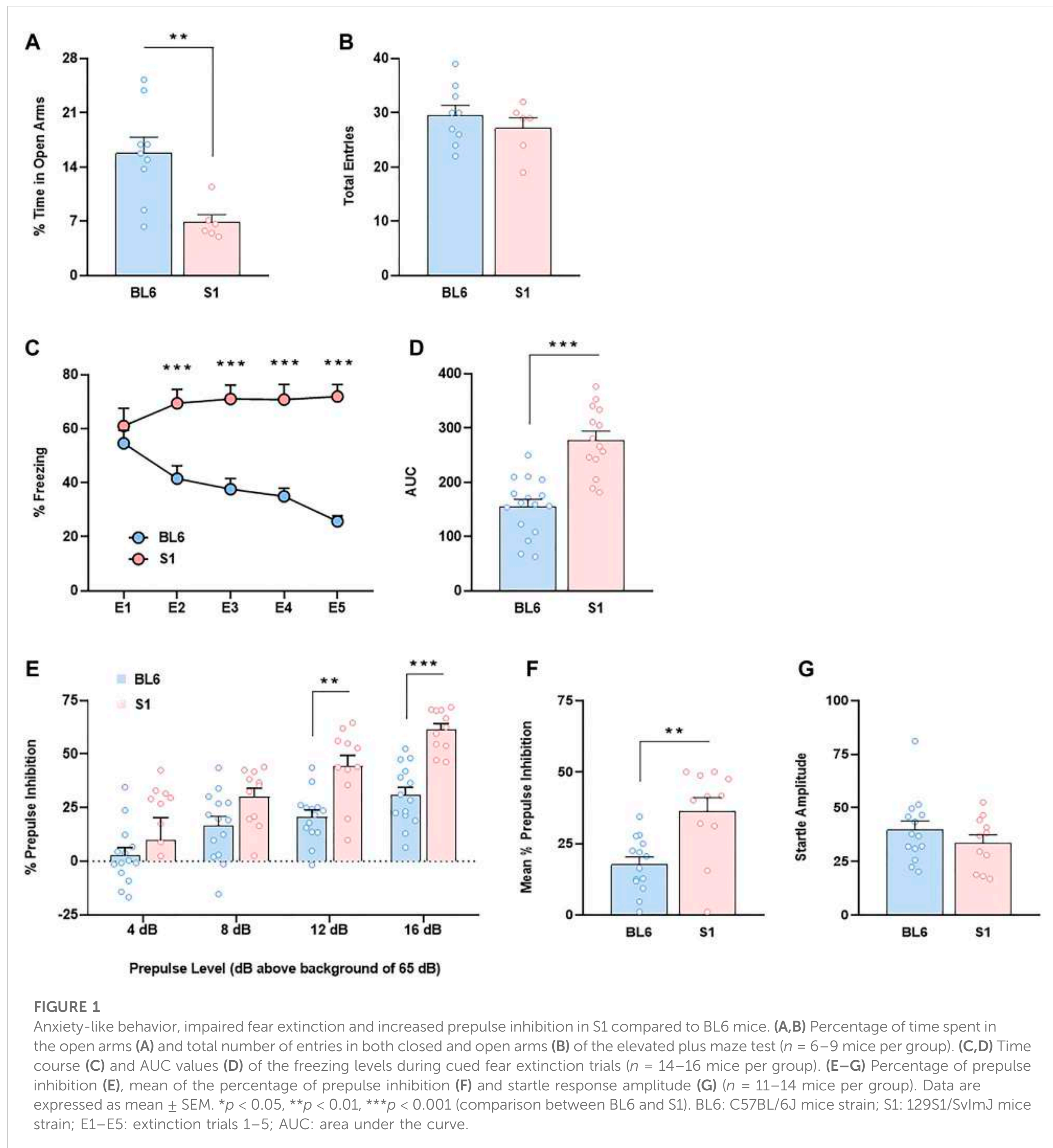

FIGURE 1
Anxiety-like behavior, impaired fear extinction and increased prepulse inhibition in S1 compared to BL6 mice. **(A,B)** Percentage of time spent in the open arms **(A)** and total number of entries in both closed and open arms **(B)** of the elevated plus maze test (n = 6–9 mice per group). **(C,D)** Time course **(C)** and AUC values **(D)** of the freezing levels during cued fear extinction trials (n = 14–16 mice per group). **(E–G)** Percentage of prepulse inhibition **(E)**, mean of the percentage of prepulse inhibition **(F)** and startle response amplitude **(G)** (n = 11–14 mice per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (comparison between BL6 and S1). BL6: C57BL/6J mice strain; S1: 129S1/SvImJ mice strain; E1–E5: extinction trials 1–5; AUC: area under the curve.

S1 mice as showed similar freezing behavior between S1 and BL6 strains in the E1 session (Figure 1C). However, as previously reported (Hefner et al., 2008; Whittle et al., 2010), fear extinction was impaired in S1 mice as revealed the increase of freezing behavior ($F_{4,116}$ = 8.95, p < 0.001) and area under the curve (AUC) (p < 0.001) (Figures 1C,D) when compared to BL6 strain. Then, we performed the PPI test to study effects on sensorimotor gating. S1 mice showed a significant increase in basal PPI ($F_{3,69}$ = 3.43, p < 0.05) in comparison with BL6 mice (Figure 1E). This effect was significant at the prepulses of 12 (p < 0.01) and 16 dB (p < 0.001) above background of 65 dB (Figure 1E). The mean PPI score was ~51% higher in S1 than in BL6 mice (p < 0.01) (Figure 1F). This effect was independent of baseline changes in startle amplitude (Figure 1G), discarding an impact of startle reaction in the PPI modifications observed.

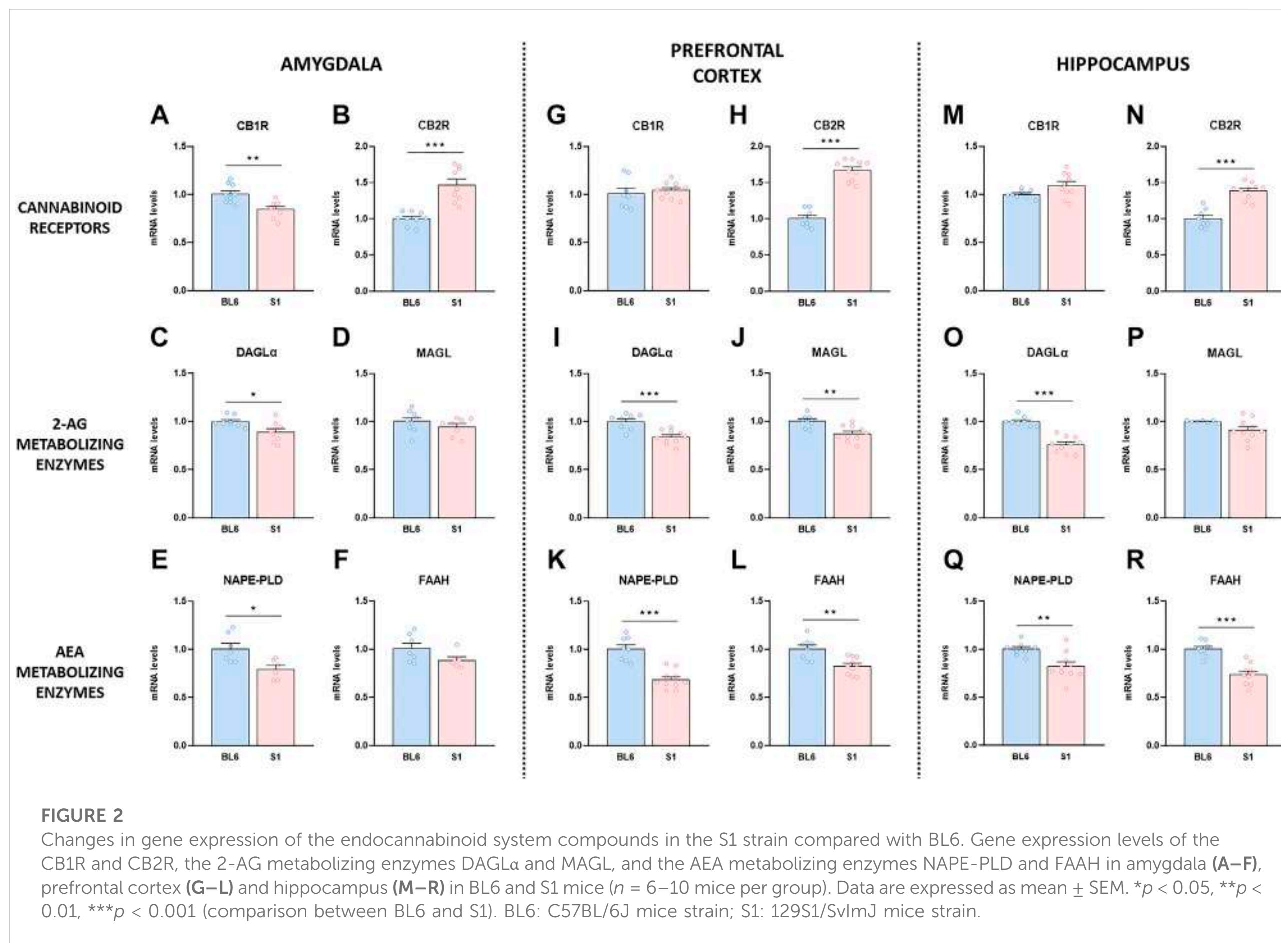

FIGURE 2
Changes in gene expression of the endocannabinoid system compounds in the S1 strain compared with BL6. Gene expression levels of the CB1R and CB2R, the 2-AG metabolizing enzymes DAGLα and MAGL, and the AEA metabolizing enzymes NAPE-PLD and FAAH in amygdala **(A–F)**, prefrontal cortex **(G–L)** and hippocampus **(M–R)** in BL6 and S1 mice (n = 6–10 mice per group). Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (comparison between BL6 and S1). BL6: C57BL/6J mice strain; S1: 129S1/SvImJ mice strain.

Increased CB2 cannabinoid receptor expression in the amygdala, prefrontal cortex and hippocampus in S1 mice

Given the role played by the ECS in the regulation of the behavioral responses altered in S1 mice, we evaluated basal gene expression of CB1R and CB2R, and the endocannabinoid-synthesizing and degrading enzymes in this mouse strain. S1 mice presented lower gene expression level of CB1R in the amygdala ($p < 0.01$) (Figure 2A), without changes either in the prefrontal cortex (Figure 2G) or the hippocampus (Figure 2M) compared to BL6 mice. Notably, quantitative RT-PCR analysis showed a robust increase of CB2R mRNA levels in the amygdala (~47%) ($p < 0.001$) (Figure 2B), prefrontal cortex (~67%) ($p < 0.001$) (Figure 2H), and hippocampus (~39%) ($p < 0.001$) (Figure 2N) in S1 mice. The expression of the enzyme in charge of 2-AG synthesis DAGLα was significantly decreased in the amygdala ($p < 0.05$) (Figure 2C), prefrontal cortex ($p < 0.001$) (Figure 2I), and hippocampus ($p < 0.001$) (Figure 2O), while MAGL expression (enzyme that degrades 2-AG) was only reduced in the prefrontal cortex ($p < 0.01$) in S1 strain (Figure 2J), with no differences in the amygdala (Figure 2D) and the hippocampus (Figure 2P). Finally, we analyzed the mRNA levels of NAPE-PLD and FAAH, the enzymes responsible for the synthesis and degradation of AEA, respectively. A decrease in the expression of NAPE-PLD was found in the three brain regions evaluated (amygdala, $p < 0.05$, Figure 2E; prefrontal cortex, $p < 0.001$, Figure 2K; hippocampus, $p < 0.01$, Figure 2Q). The expression of FAAH was significantly decreased in the prefrontal cortex ($p < 0.01$) (Figure 2L) and the hippocampus ($p < 0.001$) (Figure 2R), while no differences were observed in the amygdala (Figure 2F) in S1 mice.

Pharmacological modulation of CB2 cannabinoid receptors triggers behavioral changes in S1 mice

In view of the unexpected and strong basal increased expression of CB2R in S1 strain, we studied the consequences of the modulation of this cannabinoid receptor subtype in the phenotypic alterations previously observed in these mice. The acute administration of the CB2R antagonist SR144528 partially prevented the anxiogenic phenotype of S1 mice in the EPM test,

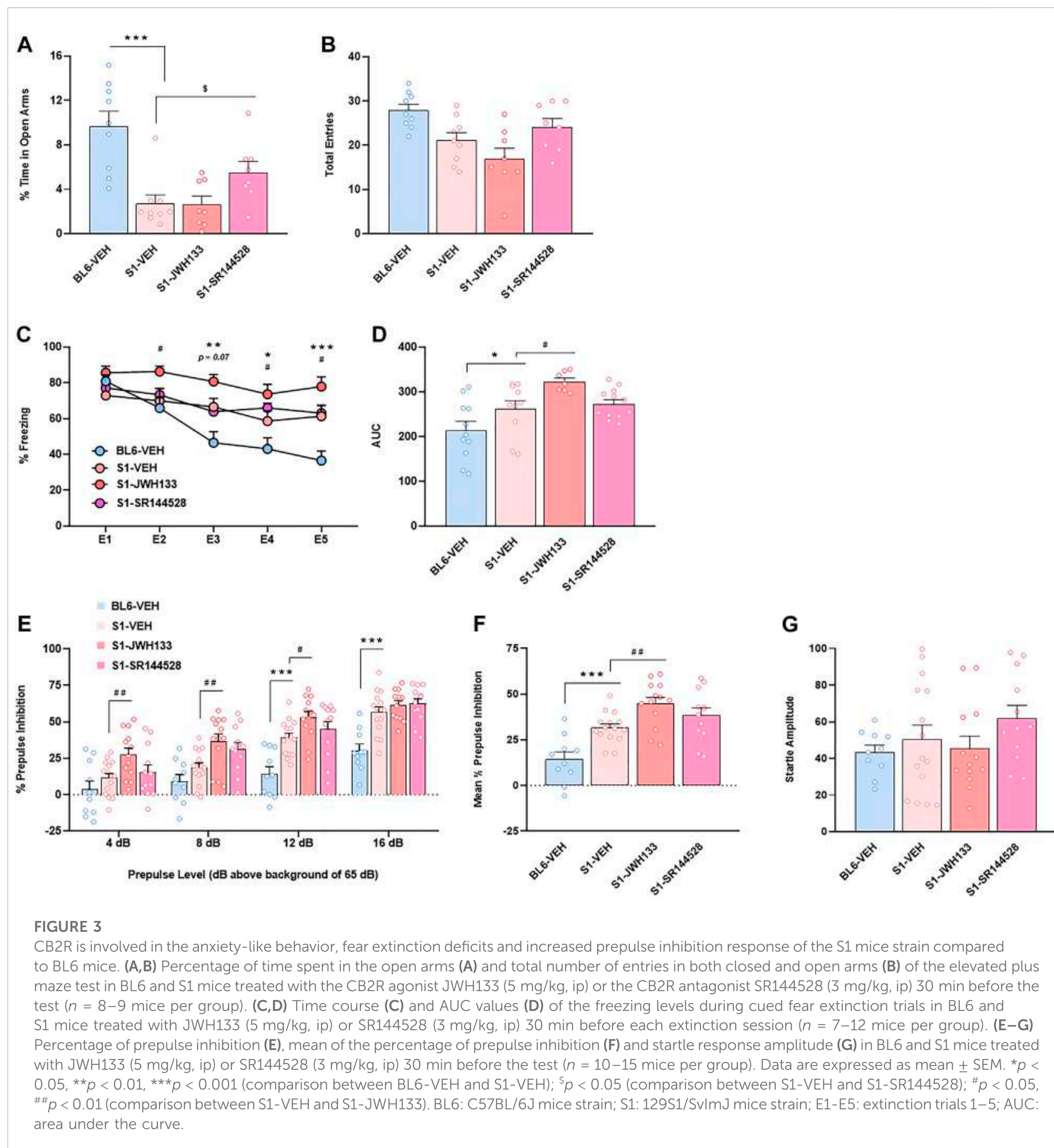

FIGURE 3
CB2R is involved in the anxiety-like behavior, fear extinction deficits and increased prepulse inhibition response of the S1 mice strain compared to BL6 mice. **(A,B)** Percentage of time spent in the open arms **(A)** and total number of entries in both closed and open arms **(B)** of the elevated plus maze test in BL6 and S1 mice treated with the CB2R agonist JWH133 (5 mg/kg, ip) or the CB2R antagonist SR144528 (3 mg/kg, ip) 30 min before the test (n = 8–9 mice per group). **(C,D)** Time course **(C)** and AUC values **(D)** of the freezing levels during cued fear extinction trials in BL6 and S1 mice treated with JWH133 (5 mg/kg, ip) or SR144528 (3 mg/kg, ip) 30 min before each extinction session (n = 7–12 mice per group). **(E–G)** Percentage of prepulse inhibition **(E)**, mean of the percentage of prepulse inhibition **(F)** and startle response amplitude **(G)** in BL6 and S1 mice treated with JWH133 (5 mg/kg, ip) or SR144528 (3 mg/kg, ip) 30 min before the test (n = 10–15 mice per group). Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (comparison between BL6-VEH and S1-VEH); $^{\$}p < 0.05$ (comparison between S1-VEH and S1-SR144528); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (comparison between S1-VEH and S1-JWH133). BL6: C57BL/6J mice strain; S1: 129S1/SvImJ mice strain; E1-E5: extinction trials 1–5; AUC: area under the curve.

as revealed one-way ANOVA ($F_{3,30} = 11.81$, $p < 0.001$) and post hoc comparison between S1 groups treated with vehicle or SR144528 ($p < 0.05$) (Figure 3A). No modification was observed in the total number of entries between these two groups (Figure 3B). Moreover, the injection of the CB2R agonist JWH133 did not alter anxiety-like behavior in S1 mice (Figure 3A). In contrast, JWH133 potentiated the resistance of cued fear extinction in S1 mice as showed the increase of freezing behavior ($F_{12,144} = 3.73$, $p < 0.001$) ($p < 0.05$ at E2, E4 and E5) and AUC ($F_{3,36} = 7.05$, $p < 0.001$) ($p < 0.05$) (Figures 3C,D) when compared to S1 mice treated with vehicle. However, the administration of SR144528 did not modify fear extinction in S1 mice (Figures 3C,D). A significant increase of PPI of startle reflex was observed by the administration of JWH133 in S1 strain ($F_{9,138} = 2.70$, $p < 0.01$) (Figure 3E). Thus, post hoc comparison revealed differences between S1 mice treated with vehicle or

JWH133 at the prepulses of 4 ($p < 0.01$), 8 ($p < 0.01$), and 12 dB ($p < 0.05$) above background of 65 dB (Figure 3E). An overall increase of PPI due to JWH133 treatment was observed when representing mean PPI score ($F_{3,46} = 13.07$, $p < 0.001$) ($p < 0.01$) (Figure 3F). However, the magnitude of startle reflex was not altered by JWH133 injection (Figure 3G). SR144528 administration did not modify PPI behavior (Figures 3E,F) nor startle amplitude (Figure 3G) in S1 mice. Taken together, these results suggest that CB2R could take part of the molecular mechanisms that underlie the phenotypic alterations of the S1 strain.

Discussion

Our data show remarkable changes in the expression levels of several components of the ECS in different brain regions in S1 mice. Particularly interesting, CB2R expression was strongly increased in the amygdala, prefrontal cortex and hippocampus in this strain compared to BL6 mice. These alterations suggest that CB2R could be involved in the phenotypic characteristics observed in S1 mice. Indeed, acute pharmacological modulation of CB2R induced behavioral alterations in important neurobiological processes in these mice. Future experiments evaluating the effects of chronic CB2R agonists and antagonists in S1 mice would be interesting since acute or chronic administration of CB2R ligands could result in different responses (García-Gutiérrez et al., 2012). The use only of male mice is a limitation of this study as several reports show evidences for sex differences in animal models of neurobehavioral disorders (Palanza and Parmigiani, 2017).

Genetic differences between strains are likely to affect several phenotypic features offering a powerful tool with which to expand our knowledge about the factors that influence psychiatric conditions. S1 inbred mice showed higher innate anxiety compared to BL6 mice as revealed the decrease of the percentage of time in open arms in the EPM test. In agreement, these mice spent significantly less time in the center of the open field (Rodriguez et al., 2020) and in the light compartment in the light-dark box (Millstein and Holmes, 2007) confirming an anxiogenic-like behavior. Cued fear extinction was impaired in S1 mice relative to the good-extinguishing BL6 strain, as previously established (Hefner et al., 2008; Whittle et al., 2010). Finally, we observed an increase of PPI of startle reflex in S1 compared to BL6 mice without modification of the startle amplitude. A high level of PPI was previously seen in S1 mice in relation to BL6 strain (Millstein et al., 2006), although no direct comparison between strains was performed in this study. Considering that the ECS participates in the regulation of anxiety (Petrie et al., 2021), extinction of aversive memories (Mizuno and Matsuda, 2021) and sensorimotor gating (Dissanayake et al., 2013), this neuromodulatory system could contribute to the phenotypic alterations observed in S1 mice. Interestingly, we found important differences in the mRNA levels of various components of the ECS between S1 and BL6 mice in several brain areas.

Our results showed a significant reduction of CB1R and NAPE-PLD expression in the amygdala of S1 strain which could in part explain the anxiogenic phenotype and resistance of fear extinction characteristic of these mice. In agreement, CB1R knockout mice are anxiogenic (Martin et al., 2002) and show strongly impaired extinction in auditory fear-conditioning tests (Marsicano et al., 2002). The recently developed NAPE-PLD inhibitor LEI-401 reduced AEA levels in the mouse brain and impaired extinction of an aversive memory in BL6 mice (Mock et al., 2020). Moreover, the systemic administration of the selective FAAH inhibitor AM3506 rescued fear extinction deficits in S1 mice (Gunduz-Cinar et al., 2013). This effect was fully recapitulated by intra-amygdala infusion of AM3506 by a mechanism involving CB1R (Gunduz-Cinar et al., 2013), consistent with the changes observed in our study. On the other hand, the expression of DAGLα also decreased in the amygdala in S1 strain. This change could be also related to the extinction deficits typical of this strain since mice deficient in DAGLα, which have reduced 2-AG brain levels, also exhibit impaired fear extinction (Jenniches et al., 2016). DAGLα, NAPE-PLD and FAAH expression were also reduced in the prefrontal cortex and hippocampus in S1 mice, brain areas which are also important mediators of fear regulation and anxiety (Maren et al., 2013). Future studies evaluating endocannabinoid levels will help to clarify the possible functional relevance of the changes in the expression of these enzymes in S1 strain.

Interestingly, CB2R expression was strongly enhanced in the amygdala, prefrontal cortex, and hippocampus in S1 related to BL6 mice. The increased expression of CB2R could be the result of a compensation of the general reduced expression of the synthesizing and metabolizing enzymes and associated possible changes in endocannabinoid levels in S1 mice. This is the case of other neurotransmission systems such as the opioid system. Thus, marked region-specific up-regulation of the mu, delta, and kappa opioid receptors was observed in mice lacking the proenkephalin and prodynorphin genes (Clarke et al., 2003). In any case, future experimental work will be necessary to establish a possible relationship between the changes in enzymes gene expression and the increased CB2R expression observed in S1 mice. Although CB2R was initially considered as a peripheral cannabinoid receptor, several recent studies suggest a role of this receptor in several neuropsychiatric disorders (Banaszkiewicz et al., 2020; Kibret et al., 2022). Contradictory results have been obtained regarding the potential function of CB2R in the regulation of anxiety-like behavior in both genetic and pharmacological studies. Deletion of the CB2R produced an anxiogenic-like response in the EPM test (Ortega-Alvaro et al.,

2011), while CB2R conditional knockout mice in dopamine neurons showed an anxiolytic-like phenotype in the same test (Liu et al., 2017). CB2R overexpression in mice decreased vulnerability to anxiety (García-Gutiérrez and Manzanares, 2011), but induced an impairment of the anxiolytic action of alprazolam. Acute treatment with the CB2R antagonist AM630 increased anxiety in Swiss ICR mice (García-Gutiérrez et al., 2012), while the administration of the CB2R agonist JWH133 did not produce any effect in the same study. Our data suggest that the increased expression of CB2R found in the amygdala, prefrontal cortex and hippocampus (brain areas involved in regulating anxiety-like behaviors) in S1 mice could be related to the anxiogenic phenotype of these mice. Thus, the acute administration of the CB2R antagonist SR144528 partially rescued the anxiogenic-like behavior in S1 mice. In this sense, chronic treatment with AM630 reduced anxiety-like behavior in the spontaneously anxious DBA/2J strain of mice (Yilmazer-Hanke et al., 2003), suggesting that this cannabinoid receptor may result a relevant target for the treatment of anxiety-like disorders. Acute effects of AM630 in anxiety were not evaluated in DBA/2J mice in this study (García-Gutiérrez et al., 2012).

S1 mice constitute a well-established model of impaired fear extinction (Hefner et al., 2008; Whittle et al., 2010). Recently, a novel role of amygdalar CB2R in the regulation of the extinction of aversive memories has been reported in mice (Ten-Blanco et al., 2022). The intra-amygdala infusion of the CB2R antagonist AM630 blocked the fear extinction deficits induced by the overactivation of the orexin/hypocretin system, while the systemic administration of the CB2R agonist JWH133 promoted fear extinction resistance (Ten-Blanco et al., 2022). Therefore, the increase in CB2R mRNA levels observed in three prototypical areas regulating fear (i.e., amygdala, prefrontal cortex and hippocampus) in S1 mice could contribute to the impaired fear extinction characteristic of this strain. Indeed, JWH133 potentiated the extinction deficits in S1 mice, although the CB2R antagonist SR144528 did not affect this behavioral response. Congruent with this, fear extinction in S1 strain was not improved by systemic treatment with the NMDA receptor partial agonist d-cycloserine, known to facilitate extinction in rodents and effective as an adjunct to exposure therapy in human anxiety disorders (Davis et al., 2006). The inefficacy of d-cycloserine or SR144528 in S1 mice could reflect usually complex molecular mechanisms driving the extinction behavior in this mouse strain (Hefner et al., 2008).

PPI of the startle reflex is a sensorimotor gating process that reduces the startling response when a weaker sensory stimulus precedes a sudden startling stimulus. An impairment in the PPI response is observed in patients with schizophrenia (Mena et al., 2016). Several studies present evidence of the contribution of CB2R to the modulation of sensorimotor gating. Thus, AM630 potentiated the reduction of PPI induced by both the NMDA receptor antagonist MK801 and methamphetamine (Ishiguro et al., 2010), although the CB2R antagonist did not affect PPI on its own. In contrast to the pharmacological data, CB2R knockout mice showed disrupted PPI at different prepulse intensities (Ortega-Alvaro et al., 2011). On the other hand, MK801-induced decrease in PPI was attenuated by the CB2R agonists JWH015 (Khella et al., 2014) and HU-910 (Cortez et al., 2022). These results are congruent with an involvement of CB2R in the enhanced PPI observed in S1 mice. The expression of CB2R was dramatically increased in the prefrontal cortex of S1 strain, a brain area directly related to the modulation of sensorimotor gating (Tóth et al., 2017). Moreover, the administration of the CB2R agonist JWH133 enhanced the PPI inhibition in S1 mice in comparison with S1 treated with vehicle at different prepulse intensities.

In summary, our data reveal important changes in the expression levels of different components of the ECS in S1 mice. Particularly significant, the increased expression of CB2R observed in this mouse strain could contribute to its behavioral alterations. Elucidating the CB2R cell type location would shed light on the role of this cannabinoid receptor in the different functions described above. However, CB2R antibody nonspecificity strongly hinders its identification (Atwood and Mackie, 2010). CB2R antagonism with SR144528 partially rescued the anxiogenic-like behavior in S1 mice. The CB2R agonist JWH133 did not enhance anxiety in S1 mice possible due to a ceiling effect, as the percentage of time in open arms was very low in these animals. On the other hand, the CB2R agonist JWH133 worsened fear extinction and enhanced PPI of startle reflex. SR144528 did not improve fear extinction or decrease PPI in S1 mice probably because of complex molecular mechanisms driving the extinction behavior or the PPI in this mouse strain, as previously suggested (Hefner et al., 2008). Our study also underlines a role for the CB2R in the regulation of important functions of the central nervous system.

Data availability statement

All data generated or analysed during this study are available from the authors on reasonable request.

Ethics statement

The animal study was reviewed and approved by the local ethical committee (CEEA-UFV) and from the Comunidad de Madrid.

Author contributions

MT-B, IP-P, and CI-L performed behavioral and molecular experiments. FB conceived the idea and funded this project. MT-B and FB wrote the manuscript. All co-authors edited and approved the final version of the manuscript.

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Conflict of interest

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

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DISCUSSION

Humans are exposed to diverse traumatic events throughout life. Loss of our loved ones, a traffic accident, or witnessing a terrorist attack, are examples of such events that remain encoded in our memory for a long time or even forever. Unlike semantic (e.g., mathematics) or procedural (e.g., motor skills) memories, emotional memory might be unconsciously retained for the whole life without efforts. Even the neuroanatomical regions and molecular pathways involved are distinct among the different types of memories (Camina and Güell, 2017). This has been evolutionary preserved, since emotions warn us about rewarding factors, as well as potential dangers for our survival (Kensinger and Murray, 2012). In this latter case, emotional memory is referred to as fear or aversive memory, which constitutes the core element addressed in this thesis.

In order to correctly overcome and extinguish fear memories, it is essential to identify risk factors that might impair fear extinction, and to better understand the underlying mechanisms regulating this process. On the one hand, genetic and epigenetic factors determine the vulnerability to develop fear-related disorders, such as PTSD, phobias and panic. In this sense, abuse drugs and/or stress are reported to be key elements for the onset of several psychiatric disorders by themselves, or in combination with other genetic or environmental factors (Assary et al., 2018; Bremner et al., 2020). For that reason, the first goal of the present thesis was to evaluate long-term effects of THC and stress exposure during the adolescence, a vulnerable life stage in which cannabis consumption is dramatically high among today's society (**Article 1**). On the other hand, it is important to increase our knowledge about the neural networks and molecular mechanisms regulating fear extinction, as potential targets may be identified to treat fear-related disorders. Therefore, the next objective of this thesis was to elucidate fear-related neural mechanisms, thus focusing on the orexin and the endocannabinoid systems given their involvement in such fear response (**Article 2**), and by testing the endocannabinoid tone in a mouse model of impaired fear extinction (**Article 3**).

To reach the aforementioned objectives, different endophenotypes were generated through multiple procedures, including chronic subcutaneous injections, stereotaxic surgeries, or breeding and maintenance of specific mouse strains, among others. Once achieved the desired phenotypes, diverse behavioural tests were employed. In particular, different fear extinction paradigms and other behavioural tasks were used. Most of the tissue samples were obtained after such experiments, when animals were sacrificed. Then, biochemical studies and the subsequent quantification with microscopy (if required) were performed. A remarkable strength of these studies was the

collaboration with other research groups that conducted biochemical experiments or provided us specific animal models, thus increasing scientific quality and validity.

1. Long-term effects of THC and stress exposure during the adolescence on fear extinction

In the last 20 years, a clear increasing trend in cannabis consumption has been observed in the European countries. The general perception of cannabis as an easily available drug, added to the controversial debate about cannabis legalization for recreational or medicinal use, has weakened public awareness of cannabis as a harmful substance. Most alarmingly, cannabis intake among adolescents and young adults aged 15-24 (19.1%) was much more than twice compared to general population aged 15-64 (7.7%), with striking sex-differences among new cannabis users (84% men, 16% women) (European Monitoring Centre for Drugs and Drug Addiction, 2022). Hence, research should pay special attention to this group of young population by evaluating long-term effects of cannabis consumption. In addition, stress is a vulnerable factor that might end up evoking symptoms related to diverse psychiatric disorders, such as anxiety, depressive or sleep-wake disorders, among others (Ventriglio et al., 2015; Yang et al., 2015; Guest and Guest, 2018; Juruena et al., 2020). A relatively common alternative to deal with stress is drug consumption with the aim of alleviating these symptoms, as also observed in animal studies (Sinha, 2008; Norman et al., 2015). Therefore, a major challenge in neuroscience research is to elucidate long-term effects of concomitant abuse drugs and stress exposure during the adolescence. In the present thesis, we addressed the effects of simultaneous adolescent THC and stress exposure on anxiety and extinction of aversive memory, in the adulthood (**Article 1**).

Adolescent cannabis consumption in humans was mimicked in mice by subcutaneous administration of increasing doses of THC, the main psychoactive compound of cannabis, during the adolescence (i.e., PND 35-49). It is difficult to establish the exact timing of adolescence in humans, and therefore it is also a complex issue in rodent models. However, it is clear that such period involves neurodevelopmental processes which give rise to a mature CNS (Schneider, 2013). An important trait of our protocol is the increasing dosage of THC administered during adolescence (3–6–12mg/kg, 5 days each dose), that was deliberately used to counter drug tolerance. Multiple studies have used a similar protocol with escalating doses of THC during the adolescent period (Llorente-Berzal et al., 2013; Cadoni et al., 2015; Garzón et al., 2021). Pure THC was administered to adolescent mice, in contrast to the numerous bioactive compounds present in cannabis preparations (Radwan et al., 2021). Despite this limitation, several studies have reported higher THC concentrations in cannabis extracts, which might

increase the total amount of THC intake and, in turn, potentiate detrimental effects (Freeman et al., 2019, 2021). On the other hand, stress procedure involved three different mild stressors (i.e., forced swimming, tail suspension and restraint) that were applied at the end of each 5-days vehicle or THC exposure. As revealed immediately after restraint, a clear stress-related physiological response was induced by increasing corticosterone plasma levels, without influence of THC treatment. Also, it is important to highlight the difference between each stressor type, in order to avoid habituation to the stressful stimuli and the subsequent HPA-axis attenuation. Two additional stressors were applied as reminders between adolescence and adulthood. These and other stressors have already been applied to evaluate the role of stress in the fear response (Maren and Holmes, 2016). Finally, THC protocol was applied to both male and female mice, given the widely-described sex differences in long-term neurobiological consequences as a result of adolescent THC consumption (Rubino et al., 2008). However, THC-stress procedure and the subsequent biochemical studies were only applied to male mice, thus revealing a clear limitation of such study.

Regarding behavioural experiments, no long-term effects were observed by adolescent THC administration in male and female mice in locomotion, anxiety-like behaviour and fear regulation. In the case of anxiety, THC treatment during adolescence has been reported to induce an anxiogenic-like behaviour in a study with adult male CD1 mice (Murphy et al., 2017), whereas lack of effect has also been observed in other studies with adult male and female rats (Rubino et al., 2008; Cadoni et al., 2015). Fear extinction of adolescent THC exposure in the adulthood had not been studied before, thus adding a novel result in this field. However, other studies addressing fear conditioning and expression (i.e., testing fear response 24 hours after cued- and contextual-fear conditioning) or passive avoidance task, had already revealed no differences between THC-treated and control mice (Rubino et al., 2009a; Ballinger et al., 2015). In accordance with these results, we found no lasting effects of THC in male and female mice in the extinction of aversive memories. Further study revealed exactly the same result in adult male and female BL6 mice exposed to orally-administered THC during the adolescence (Stollenwerk and Hillard, 2021). On the other side, stress has been commonly used as an environmental factor to induce long-term deficits in fear extinction, thus allowing the study of the underlying mechanisms involved in this response (Toledo-Rodriguez et al., 2012; Skelly et al., 2015). However, type, duration and intensity of the stressor, as well as sex, are key elements to evoke such effects (Schayek and Maroun, 2015; Deng et al., 2017; Chen et al., 2018). Our results show an anxiogenic-like behaviour in adult male mice exposed to stress during the adolescence, while no effect

was observed in the extinction of aversive memories. Surprisingly, fear extinction was disrupted in the group of adult male mice that were simultaneously exposed to THC and stress in the adolescence, thus suggesting a synergistic effect of both environmental factors to produce detrimental effects. Analogously, adult male mice were concomitantly exposed to THC and stress, and fear extinction was evaluated 3 weeks after the end of the treatment, as performed in adolescent experiments. In this case, no differences were observed between the different experimental groups. Hence, adolescence is identified as a vulnerable life stage with high risk of developing fear-related disorders, after cannabis consumption and stress exposure.

Glucocorticoid tone was analysed throughout the experiment by measuring corticosterone (cortisol in humans) plasma levels. Concomitant THC and stress exposure in adolescent male mice induced a temporal imbalance of corticosterone levels in the adulthood, thus presenting significant increased levels of such stress-related hormone immediately after the first extinction session, compared to the other groups. Despite the involvement of corticosterone in fear response, inconsistent results have been obtained regarding the role of the HPA axis in the extinction of aversive memory. Some authors have reported enhanced fear extinction as a result of the HPA-axis activation with dexamethasone (Sawamura et al., 2016; Michopoulos et al., 2017), whereas others show beneficial properties of ACTH and corticosterone attenuation in similar behavioural paradigms (Sur and Lee, 2022b, 2022a). Clearer results were obtained by analysing lasting effects of HPA axis overactivation with acute corticosterone administration in adult rats. In this case, dendritic hypertrophy in the BLA and heightened anxiety-like behaviour (as measured in the elevated plus maze test) was observed 12 days after corticosterone injection (Mitra and Sapolsky, 2008; Kim et al., 2014). For that reason, HPA axis dysfunction and the subsequent molecular alterations might contribute to the extinction deficits and changes in structural plasticity, revealed by THC- and stress-exposed mice.

After fear extinction protocol, animals were sacrificed, and brains were extracted in order to analyse the activity in the main neuroanatomical regions involved in cued-fear conditioning and extinction: BLA, IL and PL. As detailed in section *3.2.1. Neuroanatomy of fear learning and memory*, BLA presents a central role in the regulation of fear extinction, thus switching its activity to high or low fear activity depending on the cortical afferent projections. IL is responsible for fear extinction, while PL is primarily involved in the acquisition of fear memories (Sierra-Mercado et al., 2011). A hypofunction of the cortico-amygdala circuit has been widely associated to disruptions in fear extinction in numerous studies with rodent models (Hefner et al., 2008; Holmes and Singewald, 2013;

Shan et al., 2018). Conversely, increased activity of this circuit entails successful extinction, as measured by immediately-early gene expression (e.g., cFos) (Flores et al., 2014; Ganella et al., 2018; Pędzich et al., 2022). In accordance with the behavioural results previously described, adolescent THC and stress exposure induced a reduction in BLA and IL cFos expression in the adulthood, whereas PL was not modified. These biochemical results suggest long-term dysregulation of fear circuit, especially in those regions involved in the extinction of aversive memories. Brain tissues were also used to analyse structural plasticity in the BLA through dendritic spines morphology. Our results show alterations of pyramidal neurons in the BLA as a consequence of THC administration by itself, or in combination with stress. Reduced number of mushroom (mature) dendritic spines was observed in THC-treated mice, regardless of stress exposure. In agreement, previous studies have demonstrated reduced spine density in distal basal dendrites of the mPFC in THC-treated female rats during the adolescence (Rubino et al., 2015). Long-term effects of THC administration on dendritic arborization and spine density have been also observed in the mPFC, NAc and orbital frontal cortex of adult male rats (Kolb et al., 2018). Hence, THC seems to have lasting disruptive effects on neurons architecture and, in turn, functionality. On the other hand, thin (immature) dendritic spines were increased in pyramidal neurons of the BLA in adult male mice simultaneously exposed to THC and stress during the adolescence, compared to the other experimental groups. Such dendritic alterations might probably underly fear extinction deficits in this group, by prolonged impairment of the appropriate amygdalar function, one of the main areas modulating fear extinction. These findings are in accordance with previous studies associating stress-induced fear extinction deficits in male rats with dendritic retraction in the BLA (Maroun et al., 2013). Concomitant THC- and stress-exposed mice might probably present similar alterations in IL, the other major structure involved in fear extinction, which has been reported to present more immature spines in animals with poor extinction retrieval as a consequence of acute stress (Moench et al., 2015). Altogether, the interaction between THC and stress in the adolescence induces long-lasting fear extinction deficits, which are accompanied by alterations in the neuroanatomical regions involved in the regulation of such physiological response. In humans, cannabis consumption rate is high among adolescents and is commonly accompanied by stressful events. The combination of both factors increases the risk of long-term dysregulations in fear extinction, thus identifying a potential social group highly vulnerable to develop fear-related disorders in later life stages.

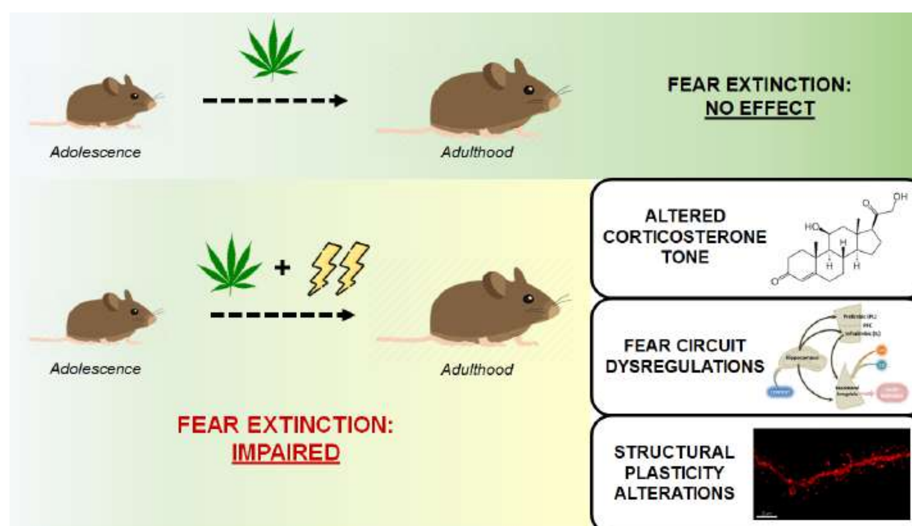

Figure 15. Schematic representation of the main findings reported in Article 1. Adolescent male and female mice treated with THC present no effects in the extinction of fear memories during the adulthood. Conversely, concomitant THC and stress exposure during the adolescent period impairs fear extinction in the adulthood. These alterations are associated with biochemical disturbances, including an altered glucocorticoid tone, dysregulations of the fear circuit, and structural plasticity alterations in the BLA.

2. Role of the orexin and the endocannabinoid systems in the underlying mechanisms regulating fear extinction

Current therapeutic approaches for fear-related disorders are based on symptom-mitigation drugs and psychological therapies. However, no pharmacological treatment has been developed to date for fear dysregulations and the subsequent clinical and neurobiological consequences. For that reason, it is important to expand our knowledge by elucidating the different players involved in such response and their underlying mechanisms. In this context, the endocannabinoid system has been widely described to play a key role in the regulation of fear (Marsicano et al., 2002), which further study with animal models of impaired fear response might open up new possibilities for therapeutic purposes. In the present thesis, the S1 mouse model of aberrant fear extinction was used to characterize and modulate the endocannabinoid system (**Article 3**). More recently, the orexin system has emerged as a potential target for fear-related disorders, since central administration of OXA impaired fear extinction in rodents, whereas pharmacological blockade of OX1R facilitated this process (Flores et al., 2014, 2017; Salehabadi et al., 2020). In human studies, a close association between orexins and fear has also been described, given the impaired acquisition of aversive memory during fear conditioning in narcoleptic individuals, which present a loss of orexin neurons (Peyron et al., 2000; Ponz et al., 2010). Conversely, increased CSF levels of OXA have been

reported in patients with panic anxiety (Johnson et al., 2010). Orexin-modulating drugs have already been approved for the treatment of sleep dysregulations without secondary warning effects, thus postulating the orexin system as a promising candidate for many other psychiatric disorders. Moreover, biochemical and functional cross-talks between the orexin and the endocannabinoid systems have been described (see section *2.5 Interaction between the orexin and the endocannabinoid systems*). Hence, it seems appropriate to focus on the endocannabinoid system as a neurobiological substrate underlying fear extinction deficits induced by orexin system dysregulation, as addressed in this thesis (**Article 2**).

Male adult mice were administered with OXA through an icv/intra-amygdala cannula, previously fixed by stereotaxic surgery. This is an intrusive intervention that requires at least 3 days of post-operative recovery before the beginning of the behavioural test, with the aim to achieve a normal behaviour and avoiding harmful effects. For that reason, both saline- and OXA-treated mice underwent exactly the same surgical procedure. Contextual-fear extinction protocol comprised a fear conditioning session on the first day, followed by 2 or 5 extinction sessions (depending on the experimental purpose) every 24 hours. In order to study the fear extinction process, preventing fear acquisition and expression bias, the different pharmacological treatments were administered after the first extinction trial. OXA or saline were infused 20 minutes after each extinction trial, thus allowing other treatments to be administered immediately after the trial and before OXA/saline. This pharmacological strategy enabled to identify OXA-underlying mechanisms depending on the treatment previously administered. Given the procedure complexity (2 hours surgery per animal, 6 days of behavioural protocol, biochemical experiments), the whole experimental research was performed by using male mice and excluding female sex, thus constituting a clear limitation in the present study (**Article 2**).

The role of the endocannabinoid system in orexin-induced impairment of fear extinction was evaluated by modulating 2-AG, since deficits in the extinction of aversive memories were found by increasing 2-AG levels through the inhibition of MAGL, unlike increasing AEA levels. Furthermore, 2-AG is synthesized after the activation of GPCR orexin receptors, as a result of the correspondent downstream molecular pathway (Turunen et al., 2012). Consequently, reduced 2-AG levels (by using the 2-AG synthesis inhibitor O7460) prevented the impaired fear extinction induced by OXA, thus suggesting that 2-AG is mediating this effect. Diverse studies have also demonstrated impaired fear extinction as a consequence of 2-AG increase (Llorente-Berzal et al., 2015; Hartley et al., 2016; Mizuno et al., 2022). In agreement with these results, OXA administration at the same dose increased 2-AG, but not AEA levels, in the amygdala 10 minutes after

the infusion. At a later time point, 2-AG levels increased in the prefrontal cortex and dramatically diminished in the hippocampus. Such observations may reflect an indirect effect of OXA in these regions and subsequent alterations in the communication between these fear-regulating structures. Indeed, functional connectivity of the hippocampus-prefrontal cortex pathway is notably modulated by the endocannabinoid system (Katona and Freund, 2012). To further clarify the role of 2-AG mediating OXA-induced deficits in fear extinction, endocannabinoid levels were analysed in the same brain areas before and immediately after the second extinction trial. Interestingly, 2-AG levels in the amygdala were significantly increased after the extinction trial in fear resistant mice treated with OXA compared to saline-treated animals, whereas no differences were observed between the same groups before this session. The same pattern was observed in the hippocampus, mainly involved in contextual cues (present protocol), but not in the prefrontal cortex, in charge of auditory cues. However, a strong correlation between 2-AG levels and impaired fear extinction was only found in the amygdala, thus indicating a central role of this area in the extinction of aversive memory. As previously mentioned, increased 2-AG levels disrupt fear extinction (Llorente-Berzal et al., 2015; Hartley et al., 2016; Mizuno et al., 2022), although the same phenotype is observed in DAGLα KO mice (Jenniches et al., 2016), and germ-free animals, both presenting reduced 2-AG levels (Chu et al., 2019; Manca et al., 2020). Therefore, optimal levels of 2-AG seem to be required to correctly extinguish fear memory. All these results considered, amygdalar 2-AG is found to be a key mediator of OXA-induced fear extinction resistance when the conditioned stimulus is evoked.

To continue deciphering the molecular pathway triggered by OXA during fear extinction, expression levels of the main endocannabinoid system components were analysed after the second extinction trial in the three aforementioned regions. Interestingly, CB2R was significantly upregulated in OXA-treated mice, only in the amygdala. In contrast, CB1R remained unchanged in animals treated with OXA among the different areas analysed. Given the controversial validity of CB2R antibodies (Atwood and MacKie, 2010; Cabañero et al., 2021), eGFP-CB2R mice became a helpful tool to quantify CB2R protein levels. These mice developed in BL6 genetic background result in the expression of GFP reporter gene under the control of the endogenous mouse CB2R promoter (López et al., 2018). According to the previous gene expression results, eGFP-CB2R mice treated with OXA and performing the same experimental protocol, reliably confirmed CB2R increased protein levels in the amygdala. Although basal CB2R expression in the CNS is low, it has been shown to be highly expressed in specific pathologies and as a consequence of several insults, given its inducible nature (Benito et al., 2003; Turcotte et al., 2016;

Robertson et al., 2017; Navarrete et al., 2021). In line with prior results, systemic or intra-amygdala administration of the CB2R antagonist AM630 blocked fear extinction resistance induced by OXA, while CB2R activation with JWH133 impaired extinction behaviour, similarly to that evoked by OXA. Despite CB2R KO mice present disrupted aversive memory consolidation in the step-down inhibitory avoidance test (García-Gutiérrez et al., 2013), the role of CB2R in fear extinction was not reported yet. These findings represent the first evidence supporting a key role of CB2R in the impairment of fear extinction, thus suggesting CB2R blockade as a new potential target for fear dysregulations. Conversely, CB1R blockade with rimonabant did not prevent OXA effect on fear extinction. This opposite role for CB1R and CB2R in the extinction of aversive memories might explain bidirectional results regarding anxiety or fear extinction studies with cannabis preparations or unselective compounds that bind both cannabinoid receptors (Raymundi et al., 2020; Bonn-Miller et al., 2021). Albeit amygdala has been shown to be in charge of CB2R effects in response to OXA, performing the same experiment in other fear-related regions would clarify the role of the fear circuitry in such process. Taken these results into account, amygdalar CB2R is confirmed to be induced along fear extinction by OXA, thus mediating its disruptive effect in the extinction of fear memory.

CB2R expression in the CNS has been broadly attributed to microglial cells, particularly in the case of neuroinflammatory processes in which microglia remains activated (Komorowska-Müller and Schmöle, 2020; Young and Denovan-Wright, 2022). Accordingly, most of the amygdalar CB2Rs of both saline- and OXA-treated eGFP-CB2R mice were localized in activated microglial cells, as revealed by immunofluorescence analysis. To further confirm this statement, microglial cells depletion with the colony stimulating factor-1 receptor inhibitor PLX5622 added to mice daily diet was observed to induce a clear decrease in CB2R, specially in OXA-treated mice presenting high levels of CB2R. Moreover, this depletion was able to reverse OXA-induced resistance to fear extinction, thus indicating microglial CB2R to probably mediate this effect. Anti-inflammatory agents might also prevent impairment of fear extinction induced by OXA, since altered microglial function and inflammation has been related to disrupted fear response (Sumner et al., 2020). Indeed, individuals with PTSD presented abnormal levels of diverse proinflammatory cytokines, including IL1β, IFNγ, IL6 and TNFα (Passos et al., 2015). Altogether, these findings involve the discovery of a novel fear extinction mechanism involving 2-AG and CB2R located in the amygdala as a consequence of the orexin system overactivation.

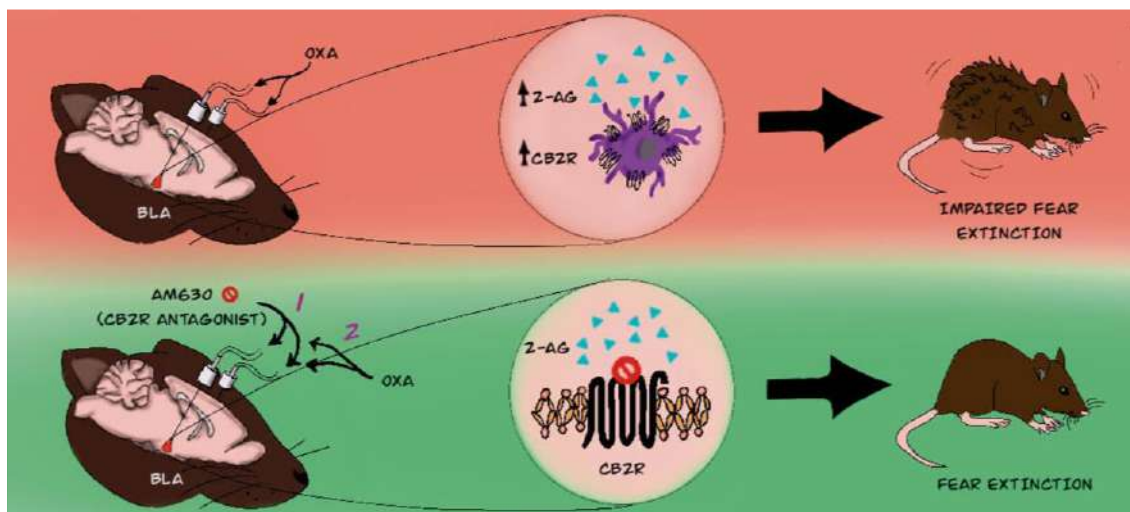

Figure 16. Schematic representation of the main findings reported in Article 2. ICV administration of OXA in male mice induces impaired fear extinction, which is associated with increased 2-AG levels and the upregulation of CB2R that seem to be present in microglial cells. Accordingly, blockade of CB2R in the amygdala before icv administration of OXA reverses these behavioural alterations. 2-AG, 2-arachidonoylglycerol; BLA, basolateral amygdala; CB2R, cannabinoid type-2 receptor; OXA, orexin-A.

Basal gene expression of CB2R was also found to be increased in the main fear-related areas (i.e., amygdala, prefrontal cortex, and hippocampus) of the inbred mouse strain S1, among many other changes in the endocannabinoid system components, compared to control BL6 mice (**Article 3**). In contrast, CB1R expression was decreased in S1 mice only in the amygdala, remaining unchanged in the prefrontal cortex and hippocampus. Interestingly, enzymes involved in the synthesis and degradation of both endocannabinoids were differentially expressed in S1 mice in comparison with BL6 mice, thus suggesting altered 2-AG and AEA levels that should be further measured. As explained in section *3.3.2. Animal models of aberrant fear response*, these mice represent a useful animal model given their behavioural traits characterized by increased stress reactivity (Singewald and Holmes, 2019; Rodriguez et al., 2020). In accordance with this feature, we observed a clear resistance to extinguish fear memories, increased anxiety-like behaviour, and alterations in the sensorimotor gating, as reported in previous studies with similar experimental approaches (Millstein and Holmes, 2007; Hefner et al., 2008; Rodriguez et al., 2020). Hence, S1 constitute an interesting tool to assess the role of CB2R in such phenotypic responses. To reach this goal, CB2R agonist JWH133 or antagonist SR144528 was systematically administered before each behavioural test. Once again, the use only of male animals constitutes a clear limitation of this study, since sex differences have been repeatedly observed in diverse animal models of neurobehavioural disorders (Palanza and Parmigiani, 2017).

In order to evaluate the role of CB2R in anxiety-like behaviour, pharmacological treatments were administered 30 minutes before the elevated plus maze test. While CB2R agonism had no effect, CB2R antagonism partially reversed the S1 anxiogenic behaviour, since significant differences were observed in comparison with vehicle-treated S1 mice, although BL6 mice still presented a greater anxiolytic-like behaviour. Inconsistent results are reported about the role of CB2R in the modulation of anxiety. In this sense, CB2R blockade with AM630 increased anxiety-like response in Swiss ICR male mice, whereas no effect was described with the CB2R agonist JWH133. However, chronic administration of AM630 induced an anxiolytic effect in spontaneously anxious DBA/2 mice (García-Gutiérrez et al., 2012). Studies with genetic mouse models also presented an anxiogenic-like behaviour in CB2R KO mice (Ortega-Alvaro et al., 2011), while conditional deletion of CB2R in dopamine neurons revealed the opposite effect (Liu et al., 2017). By adding one more confusing point, CB2R overexpression in male mice was shown to reduce vulnerability to anxiety (García-Gutiérrez and Manzanares, 2011). Our model presents increased expression of CB2R in key regions regulating anxiety-like behaviours. For that reason, blocking such receptors might explain the anxiolytic response observed in S1 mice. Future research is needed to decipher the role of CB2R in anxiety and, in turn, develop potential treatments for this psychiatric disorder.

PPI test is commonly used to measure sensorimotor gating, classically disrupted in patients with schizophrenia (Powell et al., 2009; San-Martin et al., 2020), although it is also altered in other psychiatric disorders, such as bipolar, and obsessive compulsive disorders (Hoenig et al., 2005; Kohl et al., 2013; Mao et al., 2019).To assess the role of CB2R in the naturally-increased PPI of S1 mice, acute pharmacological treatments were injected 30 minutes before the PPI test. In contrast to anxiety response, CB2R agonist potentiated S1 inhibitory response, whereas CB2R antagonist presented no effect in comparison with vehicle-treated S1 mice. These results are congruent with previous studies, since CB2R agonist JWH015, as well as HU910, reversed disruptions in PPI produced by MK801, a non-competitive NMDA receptor antagonist commonly used to decrease PPI response (Khella et al., 2014; Cortez et al., 2022). Our results present remarkable increased expression of CB2R in the prefrontal cortex, a brain structure directly involved in the regulation of the sensorimotor gating (Tóth et al., 2017). According to the reported CB2R potentiation effect in PPI, activating these receptors might increase basal-augmented PPI in S1 mice. Conversely, CB2R antagonist AM630 potentiated MK801- or methamphetamine-induced reduction in PPI, although it was not able to modify such inhibitory response on its own (Ishiguro et al., 2010). Clearer results were obtained by Ortega-Alvaro and colleagues by showing significantly decreased levels of

PPI in CB2R KO mice (Ortega-Alvaro et al., 2011). CB2R antagonist SR144528 did not modify PPI in S1 mice presumably due to robust PPI increase, compared to BL6 mice. Such PPI basal increase and the subsequent resistance to reverse this phenotype might be the consequence of complex molecular mechanisms in S1 inbred mouse strain that still remain understood.

Finally, S1 and BL6 mice underwent cued-fear conditioning and 5-days extinction protocol. As previously explained, S1 inbred mouse strain is a well-established animal model of disrupted fear extinction with unaltered fear learning or conditioning (see section *3.3.2. Animal models of aberrant fear response*). Hence, these mice constitute a helpful tool to deeply investigate the mechanisms regulating extinction of fear memories (Hefner et al., 2008; Singewald and Holmes, 2019). With the aim of studying the role of CB2R in fear extinction, treatment was administered only throughout the extinction procedure. Moreover, fear expression (first extinction trial) was not altered between the different experimental groups, thus allowing non-biased comparisons of extinction responses. Following the same pattern of PPI results, CB2R agonism potentiated basal fear extinction deficits in S1 mice, whereas no effects were observed with SR144528, compared to the vehicle-treated S1 group. As previously detailed (**Article 2**), CB2R is involved in OXA-induced impairment of fear extinction. CB2R activation with the same agonist was observed to disrupt extinction of fear memories, whereas its blockade prevented OXA effects. Therefore, increased CB2R expression in the three main areas regulating fear response might explain resistance to fear extinction observed in S1 mice. In accordance with these results, CB2R agonism potentiates such disruptive effects, although CB2R antagonism did not facilitate the extinction in S1 mice. The unknown complex molecular mechanisms underlying S1 genetic background produced a similar effect by using D-cycloserine (NMDA partial agonist known to facilitate extinction in animal models and exposure therapy in human anxiety disorders) in fear extinction protocols, since this compound failed to improve fear extinction deficits in S1 mice (Hefner et al., 2008; Sartori et al., 2016). In summary, these results support the role of CB2R disrupting fear extinction in a mouse model of aberrant fear response and the subsequent behavioural traits. However, CB2R modulation was not tested in BL6 control mice. This issue represents a clear limitation of the current investigation, although these experiments have already been addressed separately, as previously cited. The link between our two studies investigating the underlying mechanisms of fear extinction (**Articles 2 and 3**) is the evidence reported for the first time about CB2R as a key modulator of extinction fear memories. This novel function needs to be further characterized under different conditions in order to confirm the role of CB2R in fear

extinction, thus becoming a potential target for non-well treated fear-related disorders in the future.

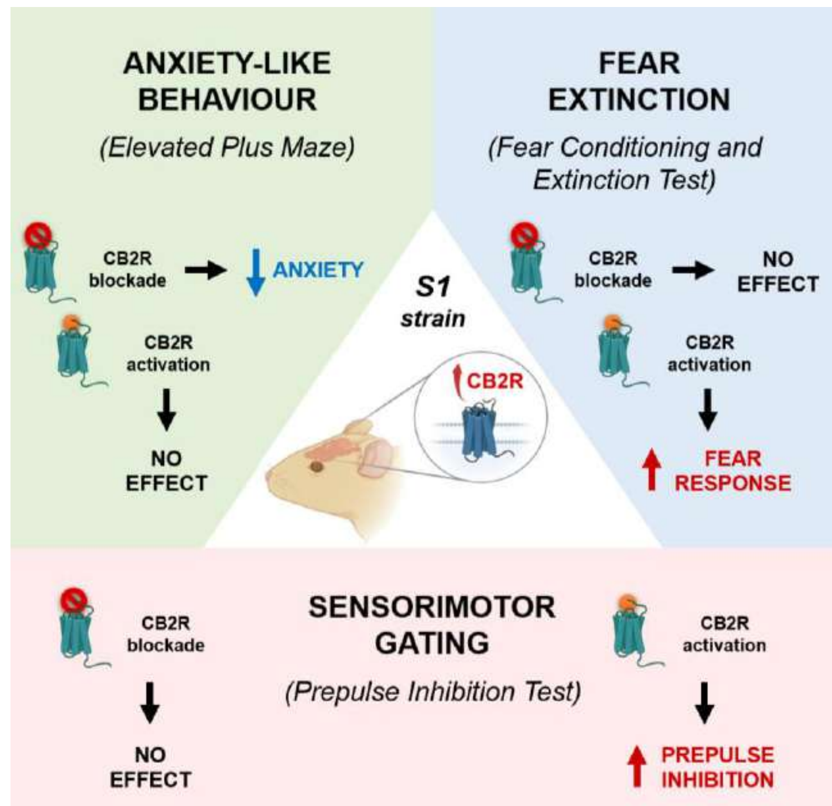

Figure 17. Schematic representation of the main findings reported in Article 3. S1 mouse model of aberrant fear extinction presents increased expression of CB2R in the main brain regions regulating fear memories, compared to BL6 mice. Hence, anxiety-like behaviour, fear extinction, and sensorimotor gating were assessed in this mouse strain by modulating CB2R, thus blocking or activating such receptor with SR144528 and JWH133, respectively. CB2R, cannabinoid type-2 receptor; S1, 129/SvImJ mouse strain.

CONCLUSIONS

The findings reported in the current thesis allow to draw the following conclusions:

1. Concomitant THC and stress exposure during the adolescent period induces fear extinction deficits in the adulthood. However, no effect is observed in both males and females by these two factors separately.
2. Such extinction impairments are associated with neurobiological disturbances in the main areas regulating fear extinction, consisting of decreased neuronal activity and structural plasticity disruptions. These alterations suggest the existence of a long-lasting dysregulation of the fear circuit.
3. Adolescence constitutes a vulnerable life stage for the development of long-term fear dysregulations, since the same protocol applied in the adulthood has no effects.
4. The endocannabinoid system is tightly involved in the underlying mechanisms modulating fear extinction deficits induced by the orexin system overactivation. In particular, 2-AG and CB2R mediate such effect.
5. CB2Rs specifically located in the amygdala are involved in the extinction deficits triggered by OXA.
6. Amygdalar CB2Rs involved in OXA-induced impaired extinction are most likely to be found in activated microglial cells, although other cell types cannot be ruled out.
7. The inbred mouse strain S1, commonly used as an animal model of aberrant fear extinction, presents increased expression of CB2R compared to C57BL/6J control mice in the amygdala, prefrontal cortex and hippocampus, the main areas regulating fear response.
8. Pharmacological modulation of CB2R induces behavioural changes in S1 mice. CB2R blockade reduces the anxiogenic behaviour, whereas CB2R activation potentiates basal-increased sensorimotor gating and fear extinction deficits.
9. CB2R emerges as a novel potential target for the treatment of fear dysregulations, thus requiring to be studied under different fear-related conditions.

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