Role of CB2 cannabinoid receptor

in nociception and food intake control

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Abstract

The endocannabinoid system is a natural modulatory system that participates in multiple physiological processes, including nociceptive, emotional and rewarding responses by fine-tuning neurotransmitter release in the central nervous system. These central responses are mainly mediated by cannabinoid receptor 1 (CB1R)-dependent mechanisms, although the side effects associated with these central responses limit the therapeutic use of CB1R agonists. Recent research on the cannabinoid receptor 2 (CB2R) provides an alternative approach to avoid the central side effects associated with CB1R stimulation. Brain CB2R expression is lower than CB1R and CB2R seems to play a neuroprotective role in response to various insults. The purpose of this Thesis was to investigate the involvement of CB2R in two different pathological conditions that currently lack effective treatment: neuropathic pain and food addiction. The results revealed that the pain-resistant phenotype of Fmr1KO mice against the nociceptive and emotional manifestations triggered by persistent nerve damage requires the participation of the CB2R. We also demonstrated that CB2R are involved in the neurobiological substrate underlying the behavioral and affective alterations that arise from food addiction. Altogether, these data highlight the potential therapeutic interest of targeting CB2R for the treatment of neuropathic pain, food addiction disorders and their co-morbid emotional manifestations.

Resumen

El sistema endocannabinoide es un sistema modulador natural que participa en múltiples procesos fisiológicos, incluidas las respuestas nociceptivas, emocionales y de refuerzo mediante el ajuste preciso de la liberación de neurotransmisores en el sistema nervioso central. Estas respuestas centrales están mediadas principalmente por mecanismos dependientes del receptor cannabinoide 1 (CB1R), aunque los efectos secundarios asociados a estas respuestas centrales limitan el uso terapéutico de agonistas de CB1R. La investigación reciente sobre el receptor cannabinoide 2 (CB2R) proporciona un enfoque alternativo para evitar los efectos secundarios centrales asociados con la estimulación CB1R. La expresión de CB2R en el cerebro es menor que la de CB1R, y CB2R parece desempeñar un papel neuroprotector en respuesta a diversas agresiones. El propósito de esta Tesis era investigar la implicación de CB2R en dos condiciones patológicas diferentes que actualmente carecen de tratamiento efectivo: el dolor neuropático y la adicción a la comida. Los resultados revelaron que el fenotipo resistente al dolor de los ratones Fmr1KO frente a las manifestaciones nociceptivas y emocionales desencadenadas por un daño nervioso persistente requiere la participación del CB2R. También demostramos que los CB2R están involucrados en el sustrato neurobiológico subyacente a las alteraciones conductuales y afectivas que surgen de la adicción a la comida. En conjunto, estos datos destacan el potencial interés de utilizar CB2R como diana terapéutica para el tratamiento del dolor neuropático, los trastornos de adicción a la comida y sus manifestaciones emocionales comórbidas.

Abbreviations

2-AG	2-arachidonoylglycerol
ACC	Anterior cingulate cortex
AEA	Anandamide
AgRP	Agouti-related protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazeloproprionic acid receptors
ARC	Arcuate nucleus
BDNF	Brain-derived neurotrophic factor
BED	Binge eating disorder
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
DA	Dopamine
DAG	1,2-diacylglycerol
DMN	Default mode network
DMS	Diagnostic and Statistical Manual of Mental Disorders
DRG	Dorsal root ganglia
ECS	Endocannabinoid system

FAAH	Fatty acid amide hydrolase
FMR1	Fragile X mental retardation gene
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GABA	Gamma-aminobutyric acid
GPCR	G protein-coupled receptor
HOMER1a	Homer scaffolding protein 1a
IASP	International Association for the study of pain
IL	Infralimbic cortex
LH	Lateral hypothalamic area
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-associated kinase
mGluR5	Glutamate metabotropic receptor 5
MSNs	Medium spiny neurons
NAc	Nucleus accumbens
NAPE-PLD	N-acylphosphatidylethanolamine phospholipase D
NArPE	N-arachidonoyl phosphatidylethanolamide
NAT	N-acyltransferase
NF-κB	Nuclear factor-kappa B
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
OEA	Oleoylethanolamine
OFC	Orbitofrontal cortex

PAG	Periaqueductal gray
PEA	N-palmitoyl ethanolamine
PFC	Prefrontal cortex
PKA	Protein kinase A
PL	Prelimbic cortex
POMC	Proopiomelanocortin
PPAR	Peroxisome-proliferator-activating receptor
PSNL	Partial sciatic nerve ligation
PVN	Paraventricular nucleus
RMV	Rostral ventromedial medulla
SNIRs	Serotonin norepinephrine reuptake inhibitors
SNpr	Sustancia nigra pars reticulata
SSRIs	Serotonin-specific reuptake inhibitors
TCAs	Tricyclic antidepressants
THC	Δ9-tetrahydrocannabinol
TNF-α	Tumor necrosis factor alpha
TRVP1	Transient receptor potential vanilloid 1
VMH	Ventromedial nucleus
VTA	Ventral tegmental area
WT	Wild-type
YFAS	Yale Food Addiction Scale

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INTRODUCTION

1 The endocannabinoid system

For centuries, the *Cannabis sativa* plant and its derivates have been used for recreational and medicinal purposes, including analgesic, anti-spasmodic, anti-epileptic, anti-emetic, and orexigenic properties, among others (Russo and Guy, 2006; Poleszak *et al.*, 2018). During the 19th century, numerous attempts were made to isolate the active compounds of the *Cannabis sativa* plant and to elucidate their structures. However, the chemical composition of the plant remained unknown until its major active component, the Δ9-tetrahydrocannabinol (THC), was isolated in 1964 (Crocq, 2020). This leading finding brought the succeeding discovery of the cannabinoid receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993) and their endogenous ligands (Devane *et al.*, 1992; Mechoulam *et al.*, 1995) in the early 1990s. Altogether these components were grouped within an endogenous modulatory system, known as the endocannabinoid system (ECS).

1.1. Components of the endocannabinoid system

The ECS comprises the cannabinoid receptors, their endogenous ligands (known as endocannabinoids), and the enzymes involved in their synthesis and degradation.

1.1.1. Cannabinoid receptors

Endogenous and exogenous cannabinoids exert their pharmacological effects through the activation of at least two main cannabinoid receptors, the cannabinoid receptor 1 (CB1R) and cannabinoid

receptor 2 (CB2R). Both are G-protein coupled receptors (GPCRs) with seven transmembrane domains mainly associated with the inhibitory Gi/o protein (McAllister and Glass, 2002) (**Figure 1**). CB1R was the first cannabinoid receptor cloned in 1990 (Matsuda *et al.*, 1990), while CB2R was cloned three years later (Munro *et al.*, 1993). Nevertheless, strong evidence supports the existence of other receptors that bind cannabinoid ligands, such as GPR55, GPR18, and GPR110 (Irving *et al.*, 2017), the transient receptor potential vanilloid type-1 receptor (TRPV1) (De Petrocellis *et al.*, 2017), and the peroxisome-proliferator-activating receptor (PPAR) (O'Sullivan, 2007).

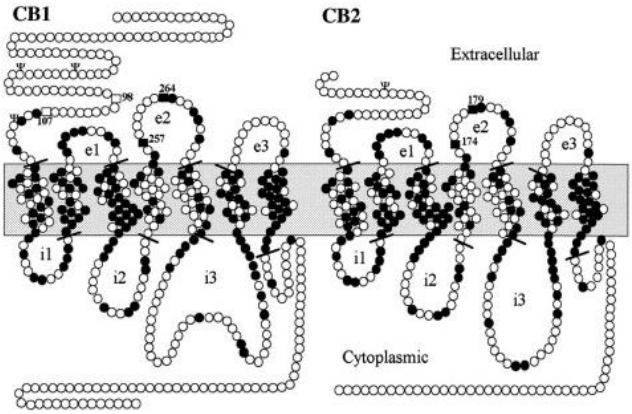

Figure 1. Schematic representation of the human CB1 and CB2 receptors. Black circles represent amino acids common to the two receptors, and white circles represent different amino acids. CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2. Adapted from (Shire *et al.*, 1996).

1.1.1.1. Cannabinoid type-1 receptor

The distribution of **CB1R** has been well characterized in humans (Westlake *et al.*, 1994b) and rodents (Tsou *et al.*, 1998). This cannabinoid receptor is abundantly expressed in the central nervous

system (CNS), particularly in the hippocampus, prefrontal cortex (PFC), basal ganglia, cerebellum, amygdala, and mesolimbic nuclei (Iannotti *et al.*, 2016) (**Fig. 2**). CB1R is known to be the most abundant GPCR in the mammalian brain. Its presence has been demonstrated in pain-processing areas such as the thalamus, periaqueductal grey (PAG), rostroventral medial medulla (RVM), and the dorsal horn of the spinal cord (Freund *et al.*, 2003) and within the mesocorticolimbic system (nucleus accumbens [NAc], ventral tegmental area [VTA], basal ganglia, lateral hypothalamus, PFC, amygdala) where CB1R modulates the hedonic aspects of eating (Parsons and Hurd, 2015). Furthermore, CB1R is also expressed in multiple peripheral tissues including the gastrointestinal tract, liver, pancreas, adipose tissue, skeletal muscle (Matias *et al.*, 2008), immune system (Jean-Gilles *et al.*, 2015), and peripheral sensory nerves (Hohmann and Herkenham, 1999), among others.

At the cellular level, CB1R is mainly located at the membrane of neuronal presynaptic terminals controlling neurotransmitter release (Mackie, 2005), especially glutamate and gamma-aminobutyric acid (GABA), but also other neurotransmitters, such as noradrenaline, dopamine (DA), serotonin, acetylcholine, and cholecystokinin (Pertwee and Ross, 2002). In addition, CB1R can form homodimers (Mackie, 2005) and heterodimers in association with other GPCRs, like CB2R (Callén *et al.*, 2012) and DA receptors D2 (Przybyla and Watts, 2010) contributing to the diversity of signaling pathways of CB1R. However, postsynaptic expression of CB1R in the hippocampus (Maroso *et al.*,

2016) and astrocytes (Navarrete and Araque, 2010; Gutiérrez-Rodríguez *et al.*, 2018) have also been described.

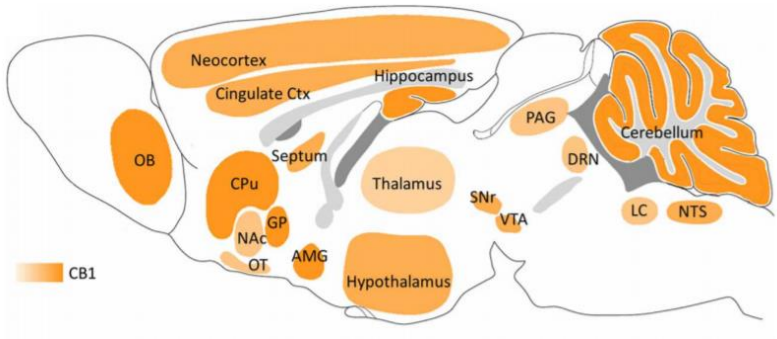

Figure 2. Schematic distribution of the main mouse brain areas containing CB1R. Color shading densities indicate the expression level of CB1R. OB, olfactory bulb; Ctx, cortex; CPu, caudate-putamen; GP, globus pallidus; NAc, nucleus accumbens; OT, olfactory tubercle; AMG, amygdala; SN, substantia nigra; VTA, ventral tegmental area; PAG, periaqueductal gray; DRN, dorsal raphe; LC, locus coeruleus; NTS, nucleus tractus solitarius. Extracted from (Flores *et al.*, 2013).

1.1.1.2. Cannabinoid type-2 receptor

CB2R is predominantly expressed in peripheral tissues, primarily present in immune cells such as macrophages, B and T lymphocytes, neutrophils, and monocytes, where it mainly mediates immune responses and anti-inflammatory properties (Svízenská *et al.*, 2008). In addition, CB2R is also present in other peripheral organs like muscle, liver, intestine, testis (Liu *et al.*, 2009), and sensory neurons (Svízenská *et al.*, 2013a). Within the CNS, the presence of CB2R has been controversial (Atwood and MacKie, 2010). It is well accepted that CB2R is mainly expressed by microglial cells in physiological states (Sánchez *et al.*, 2001), but its expression is inducible and upregulated in response

to pathological conditions (Maresz *et al.*, 2005), such as neuropathic pain (Svízenská *et al.*, 2013b). In addition, recent studies show evidence that CB2R is also expressed in neurons (Morgan *et al.*, 2009), such as DA neurons (Liu *et al.*, 2017), and mediates central responses, including depression, rewarding effects, and pain perception (Onaivi *et al.*, 2008; Shang and Tang, 2017), although the activation of this receptor seems devoid of classical psychoactive effects.

Altogether, it seems that targeting CB2R could be a promising therapeutic strategy for the treatment of several CNS alterations, avoiding the risk of centrally-mediated side effects typically associated with CB1R activation (Romero-Sandoval *et al.*, 2008).

1.1.1.3. Other cannabinoid receptors

Besides the well-known cannabinoid receptors previously presented, other receptors could also explain the effects of cannabinoid compounds that are not mediated by CB1R nor CB2R.

Among those, the **GPR55** has been classified as another member of the cannabinoid receptor family because is targeted by several cannabinoids, such as anandamide (Pertwee, 2007). GPR55 expression changes in the mouse brain due to microglial cell activation (Pietr *et al.*, 2009), and its stimulation seems to activate pain pathways (Di Marzo, 2018).

Furthermore, **GPR3**, **GPR6**, and **GPR12** are sphingosine-1-phosphate lipid receptors that have also been proposed as potential cannabinoid-like receptors (Yin *et al.*, 2009), as well as the **TRPV1** channel that bind

endogenous compounds, such as anandamide, as well as different exogenous agents, such as capsaicin (Iannotti *et al.*, 2016).

Finally, the **PPAR** also responds to several endogenous and exogenous lipids closely related to the endocannabinoids, preferentially N-palmitoylethanolamine (PEA), and N-oleoylethanolamine (OEA) (Di Marzo, 2018). PPARs exert anti-inflammatory effects by modulating the activity of several pro-inflammatory transcription factors, such as the nuclear factor-Kappa B (NF-κB) (Rakhshandehroo *et al.*, 2010).

1.1.2. Endocannabinoids

The discovery of the cannabinoid receptors suggested the presence of endogenous ligands, the endocannabinoids, and prompted their research. The first endocannabinoid identified was N-arachidonoylethanol-amide (AEA), namely anandamide (Devane *et al.*, 1992), and a few years later the 2-arachidonoylglycerol (2-AG) was discovered (Mechoulam *et al.*, 1995; Sugiura and Waku, 2000). **AEA** belongs to the N-acylethanolamin family and acts as a partial agonist to the CB1R and CB2R. Moreover, it has also affinity for TRPV1 and PPAR (Di Marzo, 2018). **2-AG** belongs to the monoacylglycerol family and its concentration in the brain is much higher than AEA (Sugiura *et al.*, 2006). 2-AG is a full agonist to CB1R and CB2R and it also activates PPAR, but not TRPV1 (Di Marzo, 2018). Other endogenous compounds could bind to CB1R and CB2R, including O-arachidonoylethanolamine (virodhamine) (Porter *et al.*, 2002), 2-arachidonoylglycerol ether (noladin ether) (Sugiura *et al.*, 1995), and N-arachidonoyldopamine (Huang and Walker, 2006), but their physiological relevance is still under study (Fonseca *et al.*, 2013). All these endocannabinoids are

long-chain polyunsaturated fatty acids derived from membrane phospholipids, specifically from the arachidonic acid, and exhibit varying selectivity for CB1R and CB2R (McAllister and Glass; Di Marzo *et al.*, 2004) (**Fig. 3**).

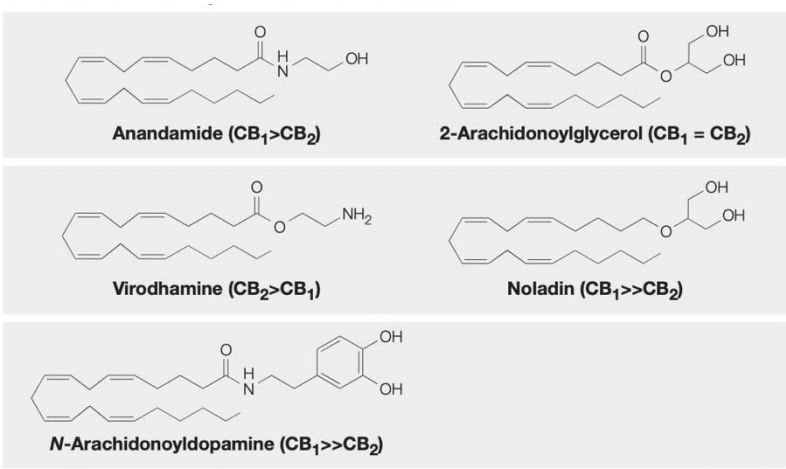

Figure 3. Chemical structures of endocannabinoids and their affinity for the main cannabinoid receptors. Chemical structures of the two best-known endocannabinoids, anandamide and 2-arachidonoylglycerol, and three putative endogenous ligands of cannabinoid receptors. CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2. Adapted from (Di Marzo *et al.*, 2004).

Both, 2-AG and anandamide are not typically stored on secretory vesicles since they are biosynthesized on demand responding to an increase in intracellular Ca^{2+} concentration. Endocannabinoids act as **retrograde messengers** (Freund *et al.*, 2003) and travel backward across the synapse regulating the release of a variety of neurotransmitters at the pre-synaptic level to prevent the presence of excessive neuronal activity (Kano *et al.*, 2009). Thus, activation of presynaptic CB1R leads to a decreased release of neurotransmitters on

excitatory or inhibitory synapses, depending on the nature of the presynaptic terminal (a process known as endocannabinoid-mediated short-term depression [eCB-STD]). This mechanism involves direct G protein-dependent inhibition of presynaptic Ca^{2+} influx (Castillo *et al.*, 2012). Moreover, endocannabinoids can also produce persistent suppression of neurotransmitter release (a process known as endocannabinoid-mediated long-term depression [eCB-LTD]). This phenomenon occurs through the inhibition of adenylyl cyclase and downregulation of the cyclic adenosine monophosphate (cAMP) / protein kinase A (PKA) pathway that ultimately inhibits the synaptic transmission (Heifets and Castillo, 2009; Kano *et al.*, 2009).

Several lipidic molecules have structural similarities with endocannabinoids, known as “endocannabinoid-like compounds”, but they do not orthosterically bind to cannabinoid receptors. Indeed, endocannabinoid-like compounds bind preferentially to TRVP1, PPAR, or GPR55 (Petrosino and Di Marzo, 2017) and comprise two large distinct families: the **N-acylethanolamines** that include N-stearoylethanolamine, PEA, and OEA, and the **2-monoacylglycerols** composed of 2-linoleoylglycerol, 2-oleoylglycerol, and 2-palmitoylglycerol (Fonseca *et al.*, 2013). Interestingly, these endocannabinoid-like compounds share some synthesis and degradation enzymes with the canonical endocannabinoids, interfering with endocannabinoid metabolism and potentiating the cannabinoid signaling. This concept has been referred to as the “entourage” effect (Ben-Shabat *et al.*, 1998; Iannotti *et al.*, 2016).

1.1.3. Enzymes involved in the biosynthesis and degradation

The bioavailability of endocannabinoids is controlled by enzymes implicated in their synthesis and degradation, which are positioned in the **synaptic cleft** (**Fig. 4**).

Both, AEA and 2-AG are arachidonic acid derivatives synthesized from precursors derived from membrane phospholipids. AEA is **synthesized** by two main enzymatic reactions: glycerophospholipids and phosphatidylethanolamine are converted into N-arachidonoyl phosphatidylethanolamide (NArPE) by a Ca^{2+}-dependent N-acyltransferase (**NAT**). Then, NArPE is hydrolyzed to AEA by the phospholipase D (**NAPE-PLD**) (Tsuboi *et al.*, 2013) (Di Marzo *et al.*, 1994). 2-AG synthesis is also a two-step process where **phospholipase C** transforms membrane arachidonic acid into 1,2-diacylglycerol (DAG). Then, DAG is hydrolyzed by either of two selective diacylglycerol lipases, DAGL-α and DAGL-β, producing 2-AG. Among them, **DAGL-α** is the main enzyme responsible for the synthesis of 2-AG in the CNS (Murataeva *et al.*, 2014).

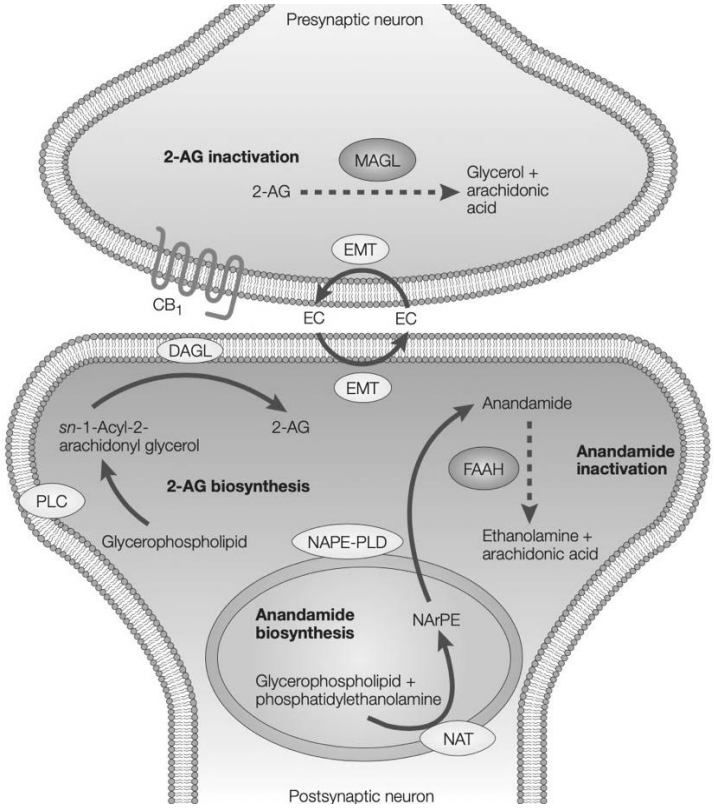

Figure 4. Synthesis and degradation of endocannabinoids. The enzymes for 2-arachidonoylglycerol (2-AG) biosynthesis are phospholipase C (PLC) and diacylglycerol lipase (DAGL), mainly localized on the membrane of postsynaptic terminals. The enzymes related to the synthesis of anandamide (AEA) are N-acyl transferase (NAT) and a specific phospholipase D (PLD), which are localized on intracellular membranes of postsynaptic neurons. This distribution supports the role of 2-AG and AEA as retrograde messengers that activate cannabinoid receptor 1 (CB1R) at the presynaptic terminals. Then, AEA is mostly inactivated on neurons postsynaptic by the fatty acid amide hydrolase (FAAH), whereas 2-AG is metabolized through the cytosolic monoacylglycerol lipase (MAGL) situated on presynaptic neurons. EC: endocannabinoid, NAPE-PLD: N-acylphosphatidyl-ethanolamine-specific phospholipase D, EMT: endocannabinoid membrane transporter, NArPE: N-arachidonoyl-phosphatidyl-ethanolamine. Adapted from (Di Marzo *et al.*, 2004).

Once AEA and 2-AG are released to the synaptic cleft and activate their target receptors, they are transported into the intracellular space and rapidly inactivated. AEA **degradation** is carried out by fatty-acid amide hydrolase (**FAAH**), whereas 2-AG is primarily metabolized by monoacylglycerol lipase (**MAGL**), both on post- and pre-synaptic neurons, respectively (Chicca *et al.*, 2017). In addition, these AEA and 2-AG metabolic enzymes are also shared with the other members of monoacylglycerol and N-acylethanolamine (Fowler *et al.*, 2017). Alternative metabolic pathways for the degradation of these endocannabinoids have also been described (Jhaveri *et al.*, 2007; Tsuboi *et al.*, 2018).

1.2. Cannabinoid intracellular signaling pathways

The stimulation of the cannabinoid receptors results in the modulation of a wide variety of cellular functions through the activation of multiple **signaling pathways** (**Fig. 5**).

As members of the GPCR family, CB1R and CB2R exert their biological function through different downstream waves. The principal pathway is mediated by activating heterotrimeric $G_{i/o}$ proteins (Gα, Gβ, and Gγ) (McAllister and Glass, 2002). Cannabinoid receptors coupling to Gαi/o leads to the inhibition of the adenylyl cyclase activity, which causes a reduction in cAMP production and PKA activity. However, coupling to heterotrimeric $G\beta\gamma_{i/o}$ proteins produces the phosphorylation and activation of different members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase 1

and 2 (ERK1/2), p38 and c-Jun N-terminal kinases (Bosier *et al.*, 2008) (**Fig. 5**).

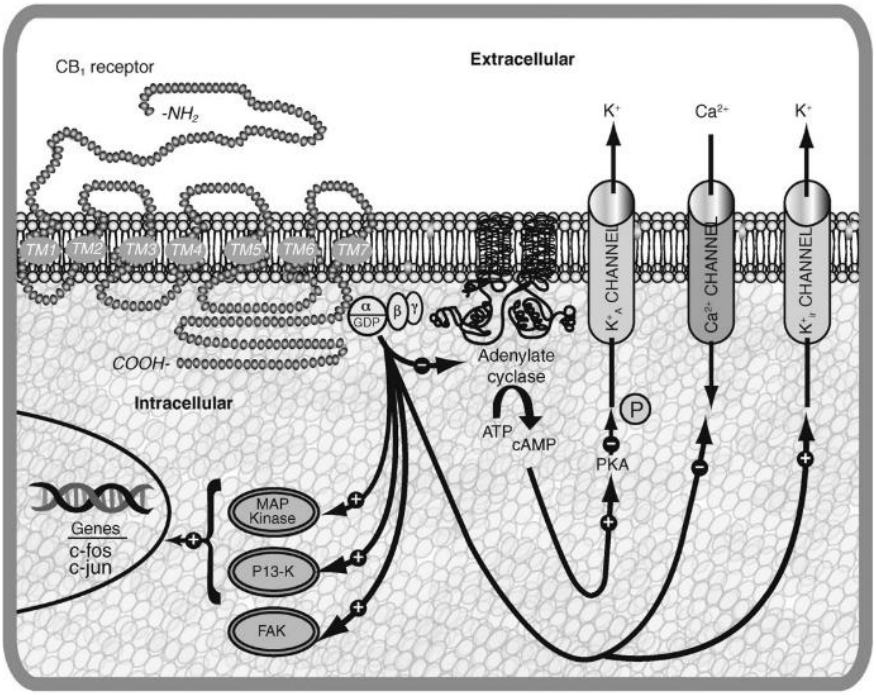

Figure 5. Major signaling pathways of cannabinoids. Activation of cannabinoid receptors results in the modulation of multiple cellular responses through distinct signaling pathways. The main one depends on G proteins. CB1R and CB2R are associated with Gαi/o producing the inhibition of the adenylate cyclase and protein kinase A (PKA) signaling. On the other hand, coupling with Gβγi/o activates the mitogen-activated protein kinase (MAPK) cascade. Moreover, CB1R regulates voltage-gated Ca^{2+} channels negatively and K^+ channels positively, thereby inhibiting neurotransmitter release. Other signaling pathways involve the activation of different effectors, such as phosphoinositide-3 kinase (PI3K) or focal adhesion kinase (FAK), that regulate gene expression. CB_1, cannabinoid receptor 1; TM, transmembrane domain; GDP, Guanosine diphosphate nucleotide; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate. Adapted from (Patel and Hillard, 2009).

In addition, stimulation of coupled $G_{i/o}$ proteins also modifies the conductance of ions inhibiting voltage-gated Ca^{2+} channels and activating A-type K^+ channels to inhibit neurotransmitter release (McAllister and Glass, 2002) (**Fig. 5**).

Cannabinoid receptor stimulation can also modulate the activation of complex protein cascades including the phosphoinositide-3-kinase (PI3K), with the subsequent activation of glycogen synthase kinase 3 (GSK-3) (Ozaita *et al.*, 2007) and mammalian target of rapamycin (mTOR) (Puighermanal *et al.*, 2009) transduction pathways.

Altogether, the response triggered by cannabinoid receptor stimulation is complex not only due to the wide range of effectors, but also to the interconnectivity between different signaling pathways.

1.3. Physiological functions of the endocannabinoid system

The endogenous presence of the ECS in multiple central and peripheral tissues implies a role in several physiological processes, including learning and memory, emotion, immune functions, psychomotor activities, feeding, reward and motivation, and pain modulation, among others (Battista *et al.*, 2012).

The ECS modulates the release of multiple neurotransmitters, such as acetylcholine, DA, GABA, histamine, serotonin, glutamate, norepinephrine, prostaglandins, and opioids. The interaction with these neuropeptides is responsible for most of the pharmacological effects of cannabinoids (Liu *et al.*, 2017).

In this thesis, we focus on the role of the ECS in the regulation of pain, emotional responses, cognition and memory, food intake and reward, which are the responses related to the main processes evaluated in this thesis.

1.3.1. Pain modulation

The ECS modulates antinociceptive responses by acting at both the peripheral and central levels through CB1R- and CB2R-dependent mechanisms.

At the **peripheral level**, CB1R is present in nociceptive sensory fibers, including C fibers, and could modulate pain transmission by inhibiting the release of peripheral neurotransmitters (Kato *et al.*, 2012). Transgenic mice lacking CB1R in nociceptive sensory neurons show increased sensitivity to heat and mechanical stimuli (Agarwal *et al.*, 2007), implying that peripheral CB1R participates in the modulation of the nociceptive responses. In contrast, CB2R is mainly present in immune system cells and exerts analgesic action at the peripheral level mainly by reducing the release of peripheral pronociceptive molecules from these cells (Ibrahim *et al.*, 2005). Moreover, both CB1R and CB2R are expressed in the dorsal root ganglia (DRG) (Starowicz and Finn, 2017).

At the **central nervous system**, the antinociceptive effects induced by cannabinoids are mainly due to the anatomical location of CB1R in the dorsal horn of the spinal cord, where it inhibits neurotransmitter release (Maldonado *et al.*, 2016). However, it has been shown that CB2R also participates in pain transmission at least at the spinal cord

level by modulating immune responses (Shang and Tang, 2017). It is now generally accepted that in the dorsal horn of the spinal cord, CB2R is mainly expressed in microglial cells in physiological states, and its expression is upregulated in response to pathological conditions, such as chronic pain (Zhang *et al.*, 2003). In addition to its spinal localization, CB1R is also located in all the major brain regions involved in pain processing and modulation, including cortex, thalamus, amygdala, hypothalamus, PAG, parabrachial nucleus, and RVM. Here, CB1R controls the transmission of nociceptive stimuli from peripheral organs to supraspinal structures, mainly at the thalamus level. CB1R-dependent analgesia also occurs by the inhibition of GABA release by RVM and PAG interneurons that facilitates descending inhibitory pathways to the spinal cord (Woodhams *et al.*, 2017). At the supraspinal level, cannabinoids can modify the emotional component of pain by modulating the neuronal activity of cortical and limbic structures, such as the amygdala (Seno *et al.*, 2018). CB2R have been identified in the central nervous system on glial cells and there is also evidence to support the expression of CB2R on subpopulations of neurons within the central nervous system, but to a lesser extent than the ubiquitously expressed CB1R. Thus, CB2R expression in DA neurons seems to modulate pain perception, as well as anxiety, depression, and rewarding effects (Liu *et al.*, 2017).

A summarized distribution of both CB1R and CB2R along pain-related regions is depicted in **Figure 6**.

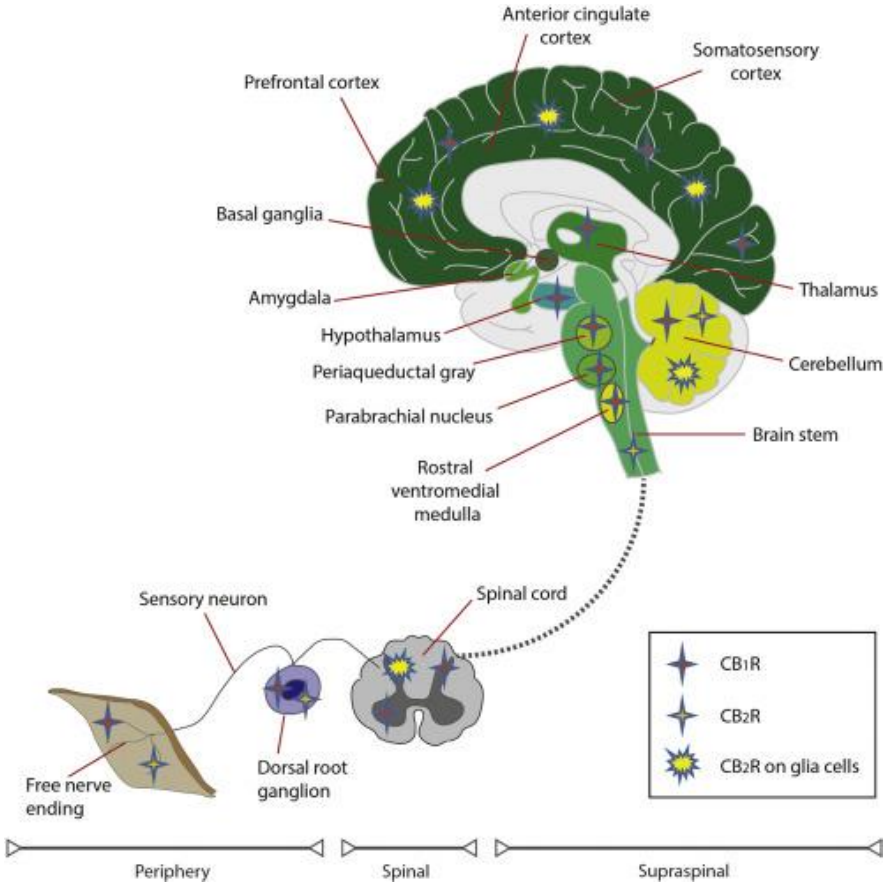

Figure 6. Cannabinoid receptor distribution throughout the pain pathway. CB1R and CB2R located at peripheral, spinal, and supraspinal sites are important modulators of the transmission and processing of pain. Extracted from (Starowicz and Finn, 2017).

1.3.2. Emotional responses

The ECS is extensively distributed in brain regions involved in the regulation of the emotional state, including the NAc, amygdala, and PFC (Jhaveri et al., 2008).

A large number of pharmacological and genetic studies support the notion of bidirectional regulation of **anxiety-like behaviors** by the ECS

(Micale *et al.*, 2013; Lutz *et al.*, 2015). Exogenous cannabinoids influence anxiety-like behavior in a biphasic manner, with low and high doses exerting anxiolytic and anxiogenic states, respectively, in both animals (Moreira and Wotjak, 2010; Lutz *et al.*, 2015) and humans (Maldonado *et al*., 2020; Mechoulam and Parker, 2013). These pharmacological effects are mainly mediated by **CB1R** (Häring *et al.*, 2012) and genetic background or environmental context can modify these responses. Thus, mice lacking CB1R showed increased anxiety-like behavior under highly aversive conditions, but not under less aversive conditions (Moreira *et al.*, 2009). Some studies performed with genetically modified mice showed that the lack of either CB1R (CB1KO) or CB2R (CB2KO) is associated with increased anxiety-like behaviors (Busquets-Garcia *et al.*, 2013; La Porta *et al.*, 2015), evidencing a direct involvement of both cannabinoid receptors in emotional responses. In agreement, the administration of a **CB2R** agonist in mice ameliorated anxiety-like behaviors in several behavioral paradigms (Bahi *et al.*, 2014). Moreover, the ECS also participates in the emotional alterations produced by neuropathic pain through CB1R and CB2R signaling. In accordance, genetic deletion of CB1R enhanced anxiety-like behaviors in mice after a peripheral nerve injury (Rácz *et al.*, 2015), whereas the administration of a CB2R agonist, JWH133, alleviated these neuropathic pain-induced emotional manifestations (Cabañero *et al.*, 2020). On the other hand, the elevation of endocannabinoid tone by administration of AEA and 2-AG or blocking of FAAH and MAGL also induces anxiolytic-like responses in rodents (Patel and Hillard, 2009; Mechoulam and Parker, 2013). These anxiolytic-like effects seem to be mediated by CB1R in the case of

elevated AEA levels, whereas CB2R seems to contribute to the effects of elevated 2-AG (Busquets-Garcia et al., 2011).

Along with the anxiolytic effects, modulation of the ECS has an impact on **depressive-like behavior** in animal models. Different cannabinoid agonists reduce depressive-like behavior via CB1R mechanisms (Umathe *et al.*, 2011; Poleszak *et al.*, 2018). However, results of CB1R and CB2R antagonism are inconsistent and even some studies showed no effect of cannabinoid receptor antagonism on depressive-like responses (Bambico *et al.*, 2007; Gobshtis *et al.*, 2007; Onaivi *et al.*, 2008). Antidepressant effects in humans have also been obtained through agonists of **CB1R**-dependent mechanisms (Patel and Hillard, 2009). In accordance, genetic deletion or inactivation of the CB1R enhances depressive-like behaviors in mice (Poleszak *et al.*, 2018). Nevertheless, the first marketed inverse agonist of CB1R (rimonabant) produced CNS-related adverse effects including depression and suicidal ideation, and thus it was withdrawn from the market despite being effective for substance use disorders and obesity (Galaj and Xi, 2019). Moreover, specific **CB2R** polymorphisms have been found associated with a certain vulnerability to autism, drug abuse, eating disorders, anxiety, and depression (Onaivi *et al.*, 2012). However, both pharmacological and genetic manipulations of CB2R revealed inconsistent results concerning its role in emotional responses (Micale *et al.*, 2013). Mice lacking CB2R display similar (Jardinaud *et al.*, 2005) or higher (Ortega-Alvaro *et al.*, 2011) depressive-like behavior than wild-type mice, depending on the strain and the experimental conditions. In this line, pharmacological administration of a CB2R

agonist in mice ameliorated depressive-like behaviors in several behavioral paradigms (Bahi *et al.*, 2014). Therefore, the possible interest of CB2R modulation in anxiety-like and depressive-like disorders still lacks experimental evidence.

1.3.3. Cognition and memory

The ECS also participates in learning and memory processes by fine-tuning synaptic plasticity in the PFC, hippocampal and amygdala networks (van Strien *et al.*, 2009; Tyng *et al.*, 2017). In **humans**, cannabis use affects several aspects of cognitive performance, including attention, working memory, verbal learning, mental flexibility, and consolidation of memories (Mechoulam and Parker, 2013). These effects are revealed after acute consumption in a dose-dependent manner. However, impairments in cognitive functions are also present beyond an acute use of cannabis (Crean *et al.*, 2011). Indeed, an acute administration of THC could induce long-term negative and psychiatric symptoms (Hindley *et al.*, 2020), highlighting the risks of cannabis use.

Similar to the findings in humans, preclinical studies demonstrated that administration of CB1R agonists produces emotional and non-emotional memory impairment in naïve **rodents** (Mechoulam and Parker, 2013). CB1R appears to be the primary cause of these central effects since its blockade with the CB1R antagonist rimonabant or CB1R genetic deletion prevents such memory deficits (Maccarrone *et al.*, 2002; Zanettini *et al.*, 2011). On the other hand, CB2R agonists have shown a beneficial or neutral effect on mice cognition, while CB2R blockage can produce cognitive impairments (García-Gutiérrez *et al.*,

2013). Nevertheless, other studies have shown no cognitive alterations when these cannabinoid receptors are blocked or absent (Busquets-Garcia *et al.*, 2013; La Porta *et al.*, 2016). Besides cannabinoid agonism and antagonism, the effects of elevating the endocannabinoid tone on memory are not clarified. Some studies showed that AEA enhancements, but not 2-AG, interfere with emotional and non-emotional memory in mice (Busquets-Garcia *et al.*, 2011), while others have reported improvements in memory performance associated with the elevation of both endocannabinoids (Pan *et al.*, 2011; Mechoulam and Parker, 2013). These controversies could be explained by the presence of CB1R-independent mechanisms since some effects are not prevented after CB1R blockade with rimonabant. According to these findings, the relationship between cannabinoid signaling and cognition is complex. However, most of the results indicate that cannabinoid agonists impair cognitive performance, while cannabinoid antagonists or genetic deletion of cannabinoid receptors improve memory (Zanettini *et al.*, 2011).

1.3.4. Reward

The ECS influences the motivation for natural rewards including palatable food, sexual activity, and social interaction, and modulates the rewarding effects of addictive drugs by acting on the DA mesocorticolimbic system (Spanagel, 2020). CB1R is richly expressed in the main structures of the mesocorticolimbic system (NAc, VTA, PFC,) (**Fig. 7**), where it can modulate promote rewarding and motivating behaviors (Manzanares *et al.*, 2018). At the cellular level, **CB1R** exerts inhibitory effects on glutamatergic and GABAergic neurons that

modulate neurotransmitter release in the VTA, NAc, and PFC (Panagis *et al.*, 2014). CB1R is present on VTA GABAergic neurons and its activation decreases the inhibitory input of GABA on DA neurons that leads to increased excitation of DA VTA neurons (D'Addario *et al.*, 2014). The activation of CB1R also decreases excitatory glutamatergic transmission of projecting neurons from the PFC to the VTA and NAc (Parsons and Hurd, 2015) (**Fig. 7**). The ECS in the mesocorticolimbic system also mediates the hedonic elements of appetite and food intake. In agreement, blockade of CB1R activity in the NAc shell (Melis and Pistis, 2007) or the VTA (Oleson and Cheer, 2012) of rodents reduces reward-seeking and food intake due to the decrease of DA release. In contrast, the activation of the CB1R increases the motivation for seeking food (Sink *et al.*, 2008). In addition, the ECS also modulates projections arising from other brain structures of the reward system, like PFC, hippocampus, and amygdala (Koob and Volkow, 2010). Finally, the ECS is also involved in the orosensorial perception of food intake, including olfaction and palatability. CB1R present in taste buds and parabrachial nucleus modulate the preferential intake of sweet and fat-rich diets than less palatable ones (DiPatrizio and Simansky, 2008; Yoshida *et al.*, 2009).

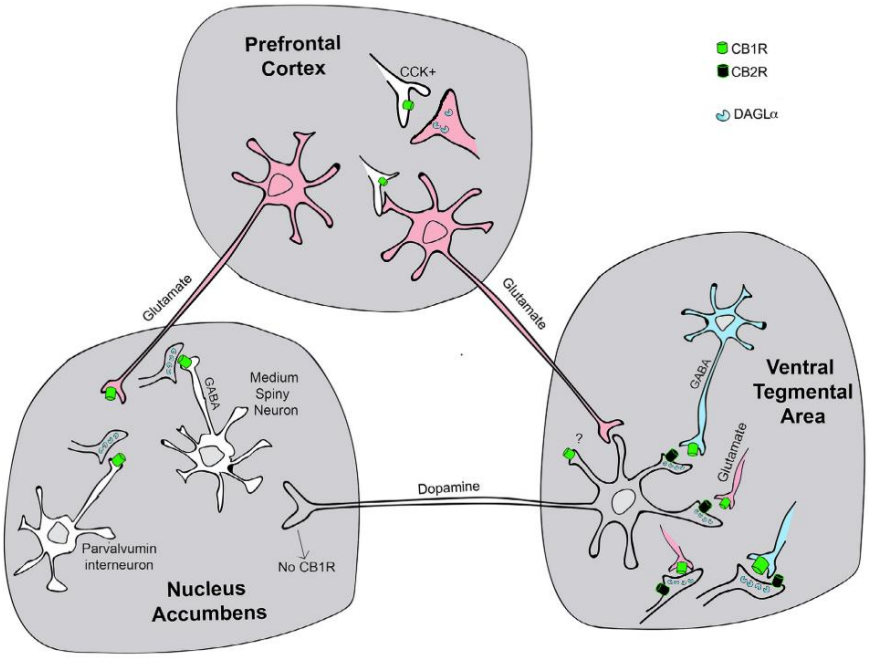

Figure 7. Localization of CB1R and CB2R in the main brain structures of the reward system. In the ventral tegmental area (VTA), CB1R are found in GABAergic and glutamatergic presynaptic terminals faced to dendrites of dopaminergic (DA) neurons. CB1R may be expressed as well in the VTA DA neurons. In the nucleus accumbens (NAc), CB1R are localized in excitatory terminals coming from the prefrontal cortex (PFC), in medium spiny neurons and parvalvumin interneurons, but not in the DA terminals. CB2R have been described in non-DA and DA VTA neurons, where its stimulation induces neuronal inhibition and reduction of DA release in the NAc. CCK+: Cholecystokinin-positive. Adapted from (Manzanares *et al.*, 2018).

It has been demonstrated that **CB2R**, as well as CB1R, is present in DA neurons of the VTA (Kano *et al.*, 2009; Liu *et al.*, 2017) modulating DA release into the NAc (**Fig. 7**), which is essential for reinforcing effects of salient stimuli. Additionally, prolonged or acute exposure to drugs of abuse increases brain CB2R mRNA expression in key reward-related

regions such as the VTA, NAc, PFC, and striatum of humans and rodents (Jordan and Xi, 2019). However, contradictory results have been reported about the rewarding effects mediated by CB2R in mice with different drugs of abuse. Thus, mice lacking CB2R showed attenuation of nicotine-seeking behavior (Navarrete et al. 2013), but increased preference for and vulnerability to ethanol consumption (Ortega-Álvaro et al., 2015). On the other hand, a reduction of cocaine and ethanol self-administration was reported in transgenic mice overexpressing CB2R (Aracil-Fernández *et al.*, 2012) and after administration of the CB2R agonist JWH133 (Navarrete *et al.*, 2018), respectively. JWH133 administration also inhibited cocaine-induced place preference in mice (Delis *et al.*, 2017), highlighting the role of CB2R in the reinforcing effects of cocaine. Moreover, deletion of CB2R seems to be a protective factor in the development of diet-induced obesity in rodents, although CB2KO and wild-type mice showed similar intake of palatable high-fat food (Deveaux *et al.*, 2009; Agudo *et al.*, 2010). However, the involvement of CB2R in the regulation of the reinforcing and motivational properties of food has not been yet fully clarified.

Altogether, these findings revealing the role of CB2R in the addictive properties of several drugs have opened a promising avenue for the clinical development of novel therapeutic approaches for substance use disorders (Jordan and Xi, 2019). More studies are needed to understand the exact implication of the CB2R in drug and food reinforcement. Thus, the work presented in this thesis intends to

provide new neurobiological evidence confirming the participation of CB2R in the reinforcing and motivational properties of palatable food.

1.3.5. Food intake

The orexigenic properties of *Cannabis sativa* compounds have been used for centuries to stimulate food intake (Bonini *et al.*, 2018). In the clinic, cannabinoid agonists have been proved effective to increase food consumption in pathological conditions related to weight loss, such as cancer or eating disorders (Di Marzo, 2018).

The effects of cannabinoids on food intake and metabolism are mainly mediated through the activation of **CB1R** at peripheral and central levels (Pagotto *et al.*, 2006), although recent studies also suggest an involvement of CB2R (Agudo *et al.*, 2010). At the **peripheral level**, the activation of CB1R triggers saving energy processes by acting on the adipose tissue, liver, skeletal muscle, pancreas, and gastrointestinal tract (Matias *et al.*, 2008) (**Fig. 8**). Endocannabinoid signaling in these peripheral organs is involved in feeding and satiety behaviors by regulating the peripheral release of peptide neurotransmitters which receptors are in the hypothalamic nuclei (Di Marzo, 2018). The main hormones affecting food intake include ghrelin, insulin, and leptin, secreted by the stomach, pancreas, and adipose tissue, respectively (Abdalla, 2017) (further described in section 3.1.1).

Energy balance is also modulated at the **central level** via CB1R activation at the brainstem and hypothalamic levels that ultimately enhances appetite by integrating the responses of peripheral peptides (Schulz *et al.*, 2021) (**Fig. 8**). In the **brainstem**, CB1R is present in

afferent and efferent neurons of the nucleus tractus solitarius which integrates signals from peripheral tissues that participate in energy homeostasis (Berthoud, 2006; Roux *et al.*, 2009). CB1R activation in the **hypothalamus** enhances appetite by stimulating orexigenic positive neurons and regulating the activity of anorexigenic positive neurons (Di Marzo and Matias, 2005; Hentges, 2005).

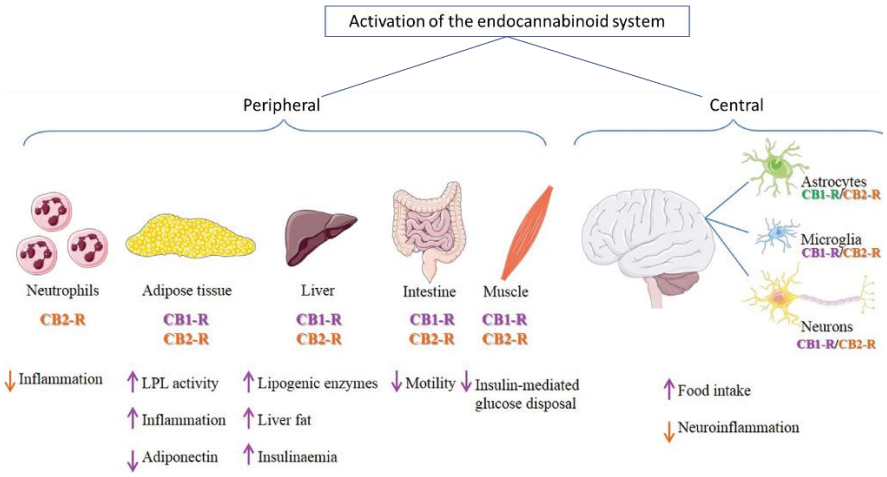

Figure 8. Role of the endocannabinoid system in food intake and energy balance. The peripheral stimulation of cannabinoid receptor 1 (CB1R) and 2 (CB2R) reduces energy expenditure acting on the adipose tissue, liver, gastrointestinal tract, and skeletal muscle. Moreover, cannabinoids can modulate food intake at a central level ultimately enhancing appetite and fat storage. LPL: Lipoprotein lipase. Adapted from (Mastinu *et al.*, 2018).

Since **CB2R** is expressed in organs involved in metabolism control, such as the liver, adipose tissue, skeletal muscle, and the endocrine pancreas, it is generally assumed that peripheral CB2R is also involved in the homeostatic control of food intake (Mastinu *et al.*, 2018) (**Fig. 8**). However, studies about the role of CB2R in this regulation have report divergent results. Some studies showed that overexpression of brain

CB2R could produce a reduction in food intake that eventually leads to a loss of body weight gain (Romero-Zerbo *et al.*, 2012). Additionally, CB2 agonists can reduce food intake in lean mice, simultaneously improving weight gain and obesity-related inflammation in diet-induced obese mice (Verty *et al.*, 2015). On the other hand, the administration of CB2R antagonists in mice also inhibits food consumption (Onaivi *et al.*, 2008). Moreover, CB2KO mice fed with a high-fat diet show less adipose tissue, hepatic inflammation, and insulin resistance in comparison to wild-type mice under similar experimental conditions (Deveaux *et al.*, 2009; Agudo *et al.*, 2010).

These findings demonstrate that both peripheral and central CB1R and CB2R regulate energy homeostasis and food intake and underline the relevance of this system as a therapeutic target to fight metabolic disorders such as binge eating and obesity (Jordan and Xi, 2019; Schulz *et al.*, 2021).

Altogether, the ECS constitutes a regulatory system crucial to maintain the homeostasis of many physiological processes. Dysregulation of this endogenous system may lead to pathological conditions, and thus, its modulation has a great therapeutic potential in multiple areas of human health.

2 Neurobiology of pain

Pain is inherent to the human being and has a protective role under physiological conditions aimed to alert external or internal stimuli that can potentially induce damage. However, pain can also represent a disastrous condition itself when it loses its warning purpose (Scholz and Woolf, 2002).

2.1 Definition

The most widely accepted definition of pain was described by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Treede, 2018). Thus, a painful experience has sensory and affective components, as well as a cognitive one reflected in the anticipation of future harm. Therefore, pain conceptualization results from the integration of two principal components (Talbot *et al.*, 2019):

- **Nociceptive** or **sensorial** component, which is a consequence of painful stimuli transmission from peripheral sensory nerves to the central nervous system. This component provides information about the location, duration, modality, and intensity of the stimuli.
- **Emotional** component, which comprises the unpleasant character of pain perception and it is influenced by previous painful experience and several psychological and social factors.

2.2 Classification

Pain has been classified in several ways according to the duration (acute, chronic), the intensity (mild, moderate, severe), the anatomical localization (cervical, spinal, pelvic,…), the etiology (rheumatism, diabetes, cancer,…) and the pathophysiological mechanisms (nociceptive, inflammatory and neuropathic) (Laird and Cervero, 1991; Woolf, 2011).

2.2.1 Based on duration

Acute and chronic pain present different pathophysiological mechanisms that underlie their distinct duration (Basbaum *et al.*, 2009). **Acute pain** is an immediate, short-lasting response to a noxious stimulus. It has a biological function as a warning mechanism, and it resolves with the healing of the injured tissue. In contrast, **chronic pain** persists beyond the damage and remains once the lesion disappears. It does not serve a biological function in most cases and it is not considered a symptom but rather a disease itself (Treede *et al.*, 2019). Moreover, persistent pain is accompanied by physical, emotional, social, or cognitive alterations that diminish the patient's quality of life (Nicholas *et al.*, 2019).

Chronic pain is not simply a temporal extension of acute pain, but rather engages highly plastic molecules and circuits (Kuner and Flor, 2017). Current theories propose that prolonged exposure to repeated nerve stimulation involves functional and structural changes at different anatomical levels of the neuronal nociceptive pathway, as

well as immune and glia participation that leads to the progression from acute to chronic pain (Maldonado *et al.*, 2016).

2.2.2 Based on pathophysiological mechanisms

According to the pathophysiological mechanisms underlying pain, it can be classically divided into nociceptive, inflammatory, and neuropathic pain (**Fig. 9**) (Cervero and Laird, 1991; Costigan *et al.*, 2009).

2.2.2.1 Nociceptive pain

Nociceptive pain is described as pain occurring in response to a brief noxious stimulus that induces minimal or no tissue damage, warning the organism of potential future harm (**Fig. 9**). The painful sensation is proportional to the intensity of the stimulus and continues as long as the noxious stimulus is present due to the normal functioning of the somatosensory nervous system (Treede, 2018). Nociceptive pain has a vital role itself since it could initiate protective reflexes that serve as normal defense mechanisms (Basbaum *et al.*, 2009).

2.2.2.2 Inflammatory pain

Inflammatory pain arises in conditions such as trauma, infections, or chronic inflammatory diseases and the injury triggers mechanisms of repair releasing inflammatory mediators that produce pain and sensitize nociceptive fibers (Woolf, 2011). Due to peripheral or central sensitization, inflammatory pain causes sensory abnormalities that alter the relationship between stimulus intensity and painful sensation (**Fig. 9**). Once the process of healing has finished, inflammatory pain

usually disappears, although in some cases it may persist leading to chronic pain, thereby losing its physiological function (Costigan *et al.*, 2009).

2.2.2.3 Neuropathic pain

Neuropathic pain is described as pain caused by a lesion or disease of the somatosensory nervous system, either in peripheral or central nervous tissue (Colloca *et al.*, 2017). Spinal lesions, diabetes, infections, chemotherapy, or chronic inflammatory diseases are examples of disorders that may cause neuropathic pain. Individuals who suffer from neuropathic pain have a diffuse pain sensation with no specific location and are characterized by the existence of spontaneous and abnormal stimulus-evoked pain (allodynia and hyperalgesia). Moreover, the relationship between the intensity of the stimulus and the painful response is disproportional becoming in some cases extremely severe and disabling for the patient (**Fig. 9**). As a consequence of the abnormal functioning of the nervous system, this type of pain is maladaptive, since it lengthens beyond the injury and remains once the lesion disappears (Costigan *et al.*, 2009).

Traditionally, neuropathic pain has been thought to be caused primarily by structural damage to the nervous tissue. However, excessive inflammation at both peripheral and central levels can play a role in the onset and management of neuropathic pain (Ellis and Bennett, 2013; Sommer *et al.*, 2018). Indeed, the inflammatory etiology of neuropathic pain is further supported since the reduction of proinflammatory mediators, such as cytokines, has been proved to

have analgesic effects in patients with spinal cord injury (Allison *et al.*, 2016).

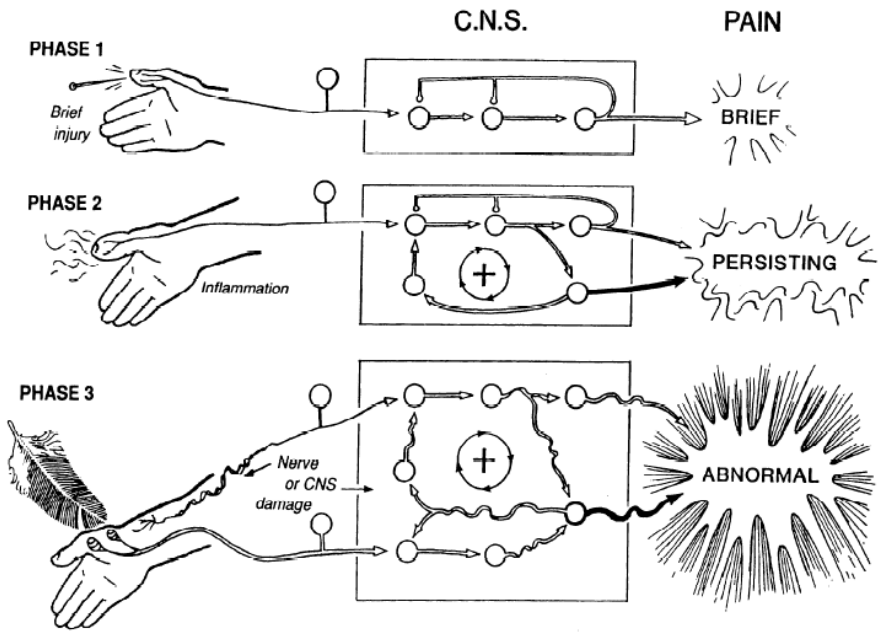

Figure 9. Models of pain processing. The nociceptive system can respond to three classical painful sensations: 1) the processing of acute noxious stimuli, 2) the consequences of prolonged noxious stimulation leading to tissue damage and inflammation, and 3) the consequences of neurological damage, including peripheral neuropathies and central pain states. CNS, central nervous system. Extracted from (Cervero and Laird, 1991).

2.3 Pain transmission

Conscious pain perception is a complex phenomenon that arises from the interaction of multiple neuroanatomic and neurochemical systems from the periphery, where the noxious stimulus is encoded, to higher nervous centres, where it is processed (Maldonado *et al.*, 2016).

2.3.1 Peripheral mechanisms

Nociceptors are high-threshold sensory nerves specialized in detecting noxious thermal, mechanical, and chemical stimuli and transducing them into action potentials (Serra Catafau, 2007; Grace *et al.*, 2014). They are located in the peripheral terminals of primary afferent neurons that have their cellular body in DRG (stimulus from the body) or the trigeminal ganglion (stimulus from the face). The peripheral axonal branch of these pseudo-unipolar neurons innervates the target organ or tissue and constitutes the sensory fibers, and the central axon synapses with second-order neurons in the dorsal horn of the spinal cord or the trigeminal nucleus caudalis (Basbaum *et al.*, 2009; Dubin and Patapoutian, 2010).

The sensory fibers are classified into four main groups considering myelinization, diameter, conduction information, and speed: Aα, Aβ, Aδ, and C fibers. However, the two types conveying pain signals under physiological conditions are Aδ and C fibers (**Table 1**). In both cases, they are free nerve endings without a clear ending receptor structure and, unlike other sensory fibers transmitting innocuous information, they show high activation thresholds and multimodal stimuli detection (Serra Catafau, 2007). **Aδ fibers** are thinly myelinated, medium (1 - 5 µm), and fast driving (5 - 30 m/s) fibers responsible for well-localized first and fast pain signals (Basbaum *et al.*, 2009). According to electrophysiological studies, they can be further divided into two categories: type I and type II fibers, which respond to acute mechanical or noxious heat, respectively (Giordano, 2005). By contrast, **C fibers** are unmyelinated and small diameter (0.2 - 1.5 µm) fibers related with slow

(2 m/s), diffuse, and long-lasting pain. According to cytochemical content, they can be divided into peptidergic and non-peptidergic fibers. Both types express TRPV1, which responds to heat and capsaicin, but only peptidergic C fibers contain peptides such as substance P and calcitonin gene-related peptide (Usoskin *et al.*, 2015). Peptidergic C fibers mainly conduct noxious thermal information, whereas non-peptidergic C fibers transmit noxious thermal, mechanical, and chemical stimuli (Basbaum *et al.*, 2009).

Fiber	Myelin	Diameter (μm)	Velocity (m/s)	Function	Dorsal horn lamina
Aα	Yes	13-20	80-120	Proprioception of skeletal muscle	III-VI
Aβ	Yes	6-12	35-75	Touch, Low threshold, Mechanoreceptors	III-VI
Aδ	Yes	1-5	5-30	Touch and temperature	I, IIo, V
				Pain, Mechanical and Cold nociceptors	I, IIo, V
C	No	0.2-1.5	0.5-2	Polymodal nociceptors (Non-peptidergic C-fibers)	IIi
				Thermal nociceptors (Peptidergic C-fibers)	I-IIo

Table 1. Primary afferent axons arriving to the spinal cord. Adapted from (Serra Catafau, 2007).

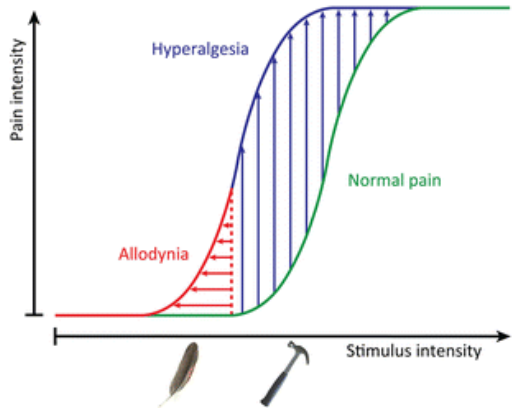

Figure 10. Sensitization to pain. This graph represents the shift from a normal pain sensation [green] to altered pain thresholds during pathological conditions. Hyperalgesia [blue] emerges from the sensitization of Aδ fibers and C fibers that become activated by low-threshold stimuli leading to an amplification of the pain signal. Allodynia [red] results from abnormal sprouting of Aβ-afferents that form new connections with nociceptive neurons changing the characteristics of response to tactile stimuli that will be perceived as painful. Extracted from (Lolignier *et al.*, 2015).

Under **physiological conditions**, Aβ fibers conduct low-threshold mechanosensitivity without eliciting pain sensation. In contrast, Aδ fibers can be activated by either peripheral mechanical, thermal, and noxious stimuli that rapidly transmit pain signals, whereas activated C fibers convey diffuse pain sensations (Serra Catafau, 2007). However, after tissue damage, there is a release of pro-inflammatory molecules from non-neuronal cells, including peptides, neurotransmitters, and cytokines that sensitize the nociceptive receptors and lead to the development of **peripheral sensitization** (further described in section 2.4.4.1). Consequently, Aδ fibers and C fibers become activated by low-threshold stimuli that lead to an amplification of the pain signal transmitted to the spinal cord, producing the so-called **hyperalgesia**

phenomenon (**Fig. 10**) (Gold and Gebhart, 2010). Furthermore, there is an abnormal growth of both Aβ and Aδ fibers to innervate the neurons receiving the lost signal from the C fibers, which ultimately changes the characteristics of response to tactile stimuli that will be perceived as painful. This phenomenon is known as mechanical **allodynia** and, as well as hyperalgesia, appears as a result of these abnormal stimulus-evoked painful sensations (**Fig. 10**) (Woolf, 2011).

2.3.2 Central mechanisms

2.3.2.1 Sensory transmission in the spinal cord

The nociceptive information gathered in the periphery travels along the primary afferent fibers, whose soma are located in the DRG, and enters into the dorsal horn of the spinal cord, which is organized into different **laminae**. Aδ and C fibers project to second-order neurons in laminae I, II, and V. Peptidergic C fibers mostly terminate in laminae I and outer II, whereas non-peptidergic C fibers synapse with second-order neurons in inner lamina II. By contrast, low-threshold Aβ fibers predominantly innervate laminae III, IV, and V (**Fig. 11**) (D'Mello and Dickenson, 2008; Basbaum *et al.*, 2009).

Most of the primary afferents use excitatory amino acids, such as glutamate and aspartate, as main neurotransmitters to activate second-order neurons. Nevertheless, neuropeptides (substance P and calcitonin gene-related peptide) and purines (ATP) act as co-transmitters in peptidergic and non-peptidergic nociceptors, respectively, to enhance pain transmission (Basbaum *et al.*, 2009). The listed neuromodulators are over-expressed in response to persistent

nociceptive inputs from primary afferent neurons contributing to the generation and maintenance of **central sensitization** at the spinal level. Consequently, neurons in the dorsal horn spinal cord exhibit a state of hyperexcitability that leads to the amplification of nociceptive signals and the emerge of allodynia and hyperalgesia phenomena (Latremoliere and Woolf, 2009).

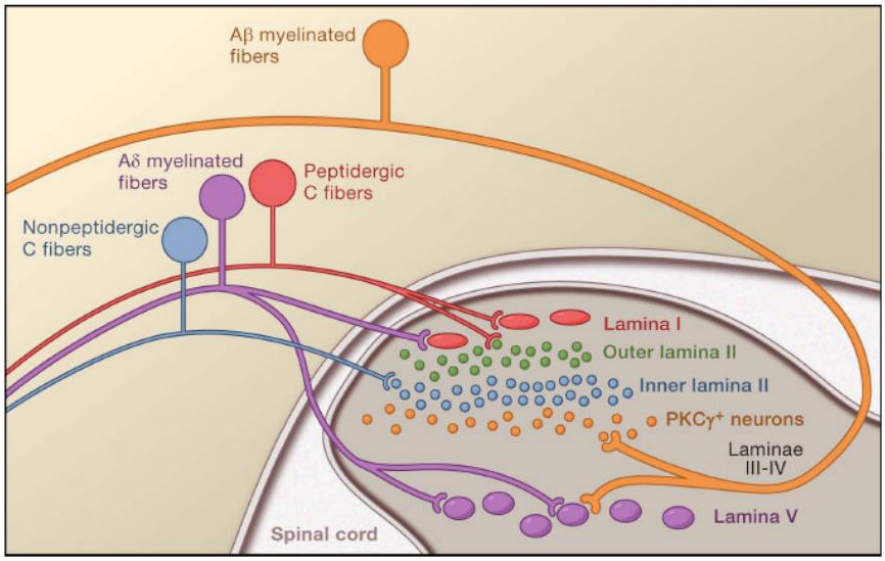

Figure 11. Termination sites of Aδ, peptidergic, and non-peptidergic C fibers in the spinal cord. Primary afferent fibers (Aβ, Aδ, and C) transmit impulses from the periphery, through the dorsal root ganglia, and into the dorsal horn of the spinal cord. The unmyelinated, peptidergic C [red] and myelinated Aδ nociceptors [purple], terminate most superficially, targeting projection neurons [red] located in lamina I. The unmyelinated, non-peptidergic nociceptors [blue] synapse upon interneurons [blue] of lamina II. By contrast, innocuous input carried by myelinated Aβ fibers (yellow terminates) synapse interneurons in the ventral half of the inner lamina II. A second set of projection neurons within lamina V [purple] receive convergent input from Aδ and Aβ fibers. Extracted from (Basbaum *et al.*, 2009).

2.3.2.2 Ascending pathways and supraspinal processing

The painful signals received in the dorsal spinal horn are carried by spinal projections of **second-order neurons** to supraspinal areas through different ascending pathways. Axons of these projecting neurons decussate at spinal level to the contralateral side and project the nociceptive information directly to thalamic structures (**spinothalamic tract**) or indirectly synapsing in brainstem nuclei, including the reticular formation (**spinoreticular tract**) or the parabrachial nucleus and PAG (**spinomesencephalic tract**) (McCarberg and Peppin, 2019) (**Fig. 12**). These three tracts constitute the anterolateral system, the main ascendant pathway involved in the transmission of nociceptive information to higher brain areas (Scholz and Woolf, 2002).

At supraspinal level, the main regions that are activated by nociceptive inputs and participate in **pain perception** are the thalamus, the somatosensory cortices, the anterior cingulate cortex (ACC), the insular cortex, the PFC, and limbic areas, such as the amygdala and NAc (Maldonado *et al.*, 2016) (**Fig. 13**). These cortical and subcortical structures have been included in the so-called “pain matrix” and may play a role in the conscious awareness and emotional aspect of the pain experience (Neugebauer *et al.*, 2004; Garcia-Larrea and Peyron, 2013).

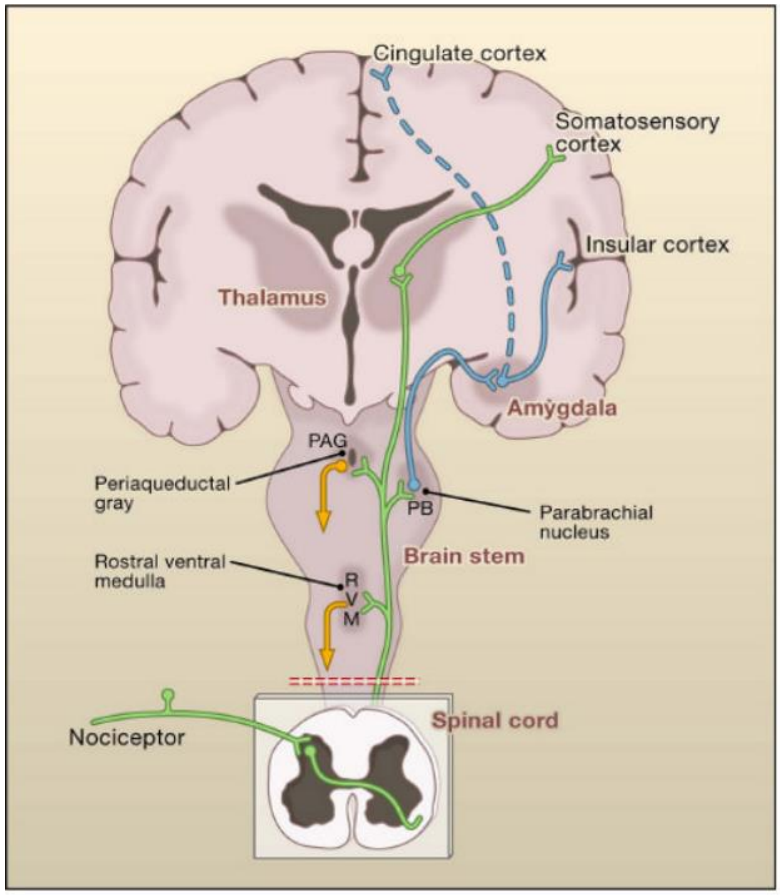

Figure 12. Main ascending and descending pain pathways. Primary afferent nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information to the somatosensory cortex through the spinothalamic tract (green line), providing discrimination information about the painful stimulus. Other projection neurons engage the spinomesencephalic tract that ends in cingulate and insular cortices via connections through the parabrachial nucleus (PB) and amygdala (blue line), contributing to the affective component of the pain experience. The amygdala and the hypothalamus project to the periaqueductal grey (PAG) and rostroventral medial medulla (RVM), where descending inhibitory pathway (yellow line) ultimately targets the dorsal horn spinal cord to modulate nociceptive inputs. Extracted from (Basbaum *et al.*, 2009).

The **thalamus** is not only a relay centre but is involved in the processing and sending nociceptive information to other brain areas (Obara *et al.*, 2013b). Thalamic projections coming from the ascending direct pathway conduct tactile, proprioceptive, and nociceptive signals to the **somatosensory cortex**, which integrates the sensory characteristics of pain, which include location, intensity, and duration of the stimulus (Thompson and Neugebauer, 2019). The thalamus also projects to cortical areas including the **PFC**, **ACC, insular cortex,** and limbic structures, such as the **amygdala** and **NAc**, implicated in the cognitive and affective-motivational components of pain (Groh *et al.*, 2017) (**Fig. 13**). The subsequent activation of the PFC and, in particular of the medial PFC (**mPFC**), is associated with the voluntary control of emotional suffering (Apkarian *et al.*, 2011). The **ACC** is linked to the cognitive-evaluative processing and the aversiveness of ongoing pain, whereas the **insular cortex** is related to both the sensory and the cognitive aspects of pain perception (Thompson and Neugebauer, 2019). The **amygdala** receives nociceptive inputs from the thalamus, the cortex, and the parabrachial nucleus (indirect ascending pain pathway), contributing to the affective dimensions of pain (Bushnell *et al.*, 2013; Neugebauer, 2015; Thompson and Neugebauer, 2019). Particularly, the neural circuit between basolateral amygdala and PFC is crucial for decision-making based on reward expectancy, risk anticipation, and punishment avoidance (Koob and Volkow, 2010). Finally, spinoreticular and spinomesencephalic tracts (indirect ascending pain pathways) send collaterals to several areas related to vegetative and homeostatic processes, such as the reticular formation, PAG, hypothalamus, and superior colliculus (tectum), involved in

autonomic responses secondary to pain (Serra Catafau, 2007). Especially, the hypothalamus receives nociceptive signals from the spinomesencephalic tract and relayed in the amygdala, which activates the hypothalamic-pituitary-adrenocortical axis and promotes the release of neuroendocrine stress hormones, including corticotropin-releasing hormone (CRH), adrenocorticotropic hormone, and cortisol (Hannibal and Bishop, 2014).

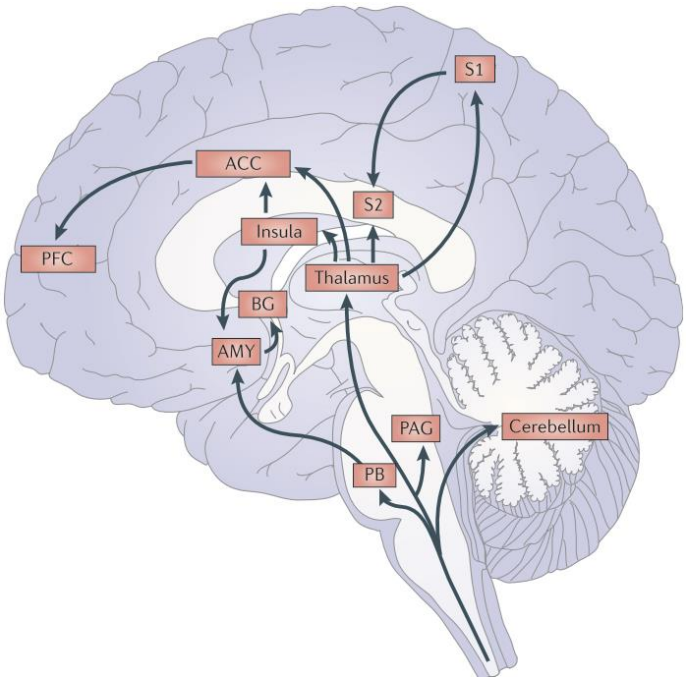

Figure 13. Supraspinal pain processing. Afferent nociceptive information enters the brain from the spinal cord. Afferent spinal pathways include the spinothalamic, spinoparabrachio-amygdaloid, and spinoreticulo–thalamic pathways. Nociceptive information from the thalamus is projected to the insula, anterior cingulate cortex (ACC), the primary (S1) and secondary (S2) somatosensory cortex, whereas information from the amygdala (AMY) is projected to the basal ganglia (BG). PAG, periaqueductal grey; PB, parabrachial nucleus; PFC, the prefrontal cortex. Extracted from (Bushnell *et al.*, 2013).

There is rising evidence for functional and structural adaptative changes of these supraspinal structures during chronic pain states that affects pain experience and will be discussed in section 2.4.4.2.

2.3.2.3 Descending pathways

Once the nociceptive information is processed in brain areas, the descending modulatory circuit can build a physiological response to modulate the painful sensation. This descending control of pain arises from several supraspinal sites, including PAG, parabrachial nucleus, medullary reticular formation, and RVM, which eventually balance the **descending facilitation** or **inhibition** of the nociceptive input in the spinal cord (Bushnell *et al.*, 2013). The classical analgesic descending system is the PAG-RMV-dorsal horn pathway, which is contained in the dorsolateral funiculus (**Fig. 14**). The PAG-RVM circuit integrates information from higher brain centers involved in the emotional and cognitive aspects of pain. Thus, the descending pathway modulates pain thresholds as a response to attention, emotions, context, and expectations, allowing a quick adaptation to the environmental circumstances (Colloca *et al.*, 2017). This descending spinal tract starts in the **PAG** which receives projections from the thalamus, hypothalamus, amygdala, and cortical areas such as the PFC. Neurons in the PAG project downstream to the **RVM**, which also receive nociceptive information from the thalamus, the parabrachial area, the locus coeruleus, and the parabrachial tract. The RMV is considered the final common relay in descending modulation of pain before sending outputs to the dorsal horn of the spinal cord (Purves *et al.*, 2012). The two main cell subpopulations from the RVM responsible to inhibit or

facilitate pain perception at dorsal horn level are OFF- and ON-cells, respectively (Chen and Heinricher, 2019).

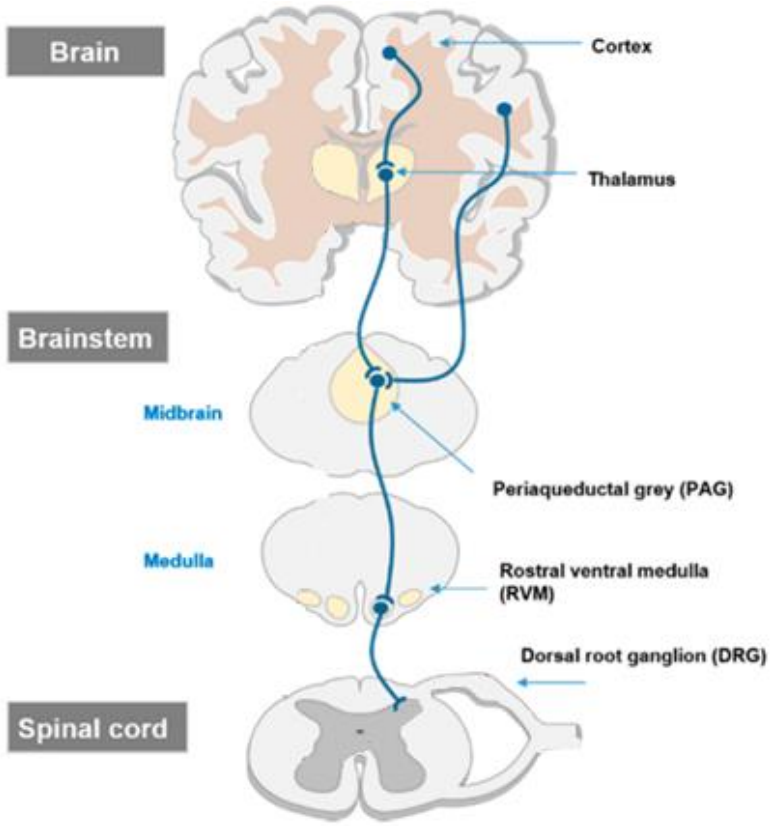

Figure 14. Simplified depiction of ascending and descending pain signaling pathways. Afferent nociceptive input enters the spinal cord via the dorsal root ganglia (DRG). Secondary order projection neurons ascend in the contralateral spinothalamic [red] tract that relays the signal to the thalamus and cortical centers. Descending pathways [blue] modulate pain transmission projecting from the periaqueductal gray (PAG) in the midbrain and the rostral ventromedial medulla (RVM) to the dorsal horn. Adapted from (Cioffi, 2018).

Moreover, local circuits within the dorsal horn also play a role in modulating the painful sensation. The “gate control theory of pain”,

exerts as an example of these regulatory spinal circuits (Melzack and Wall, 1965). This theory states that the activation of primary afferent Aβ fibers can act on local interneurons to inhibit the transmission of information from C fibers to the dorsal horn projection neurons. This explains how a mechanical stimulus, such as pressing the injured area, can temporarily ease the pain sensation.

2.4 Neuropathic pain

2.4.1 Definition and classification

Neuropathic pain is defined by the IASP as "an unpleasant sensory and emotional experience initiated by a lesion or disease of the somatosensory nervous system" (Treede *et al.*, 2008). It is characterized by spontaneous pain and abnormal responses to painful stimuli, such as hyperalgesia and allodynia (Scholz *et al.*, 2019). These troubling pain sensations are often accompanied by anxiety, depression, and impaired cognitive functions that diminish the quality of life of patients (Descalzi *et al.*, 2017).

Neuropathic pain classification is a subject under discussion. Traditionally, it can be classified according to the etiology of the insult to the nervous system, as well as the presumed location of the nerve injury (peripheral or central) (**Table 2**) (Cousins *et al.*, 2010).

Etiology	Location	
Trauma	**Peripheral**	**Central**
Ischemia or hemorrhage	Nerve	Spinal
Inflammation	Plexus	Brainstem
Neurotoxic	Dorsal root ganglia	Thalamus
Neurodegeneration	Root	Cortex
Metabolic		
Paraneoplastic		
Cancer		

Table 2. Classification of neuropathic pain. Adapted from (Cousins *et al.*, 2010).

2.4.2 Epidemiology

Neuropathic pain affects millions of people worldwide, although it is usually underdiagnosed and undertreated (Colloca *et al.*, 2017). This clinical entity has pathogenic mechanisms that remain largely unknown and current treatments are limited by the lack of efficacy and important side effects (Bouhassira and Attal, 2018). Thus, neuropathic pain represents a challenge to health care and a large economic burden for society, estimating a cost of billions of euros costs for the European population (Breivik *et al.*, 2013).

The exact prevalence of neuropathic pain is also undefined. According to general European population studies, 7-8% of adults currently have chronic pain with neuropathic characteristics (Torrance *et al.*, 2006; Bouhassira *et al.*, 2008), but the prevalence is even higher in specific subpopulations suffering other pathological conditions (**Table 3**). Furthermore, neuropathic pain is more frequent in women, aged people and most commonly affects the lower back, limbs, and neck (Colloca *et al*., 2017).

Epidemiology	Percentage	References
General Population	7-8%	Bouhassira et al., 2008; Torrance et al., 2006
Specific population		
Postsurgical herniotomy	10%	Aasvang et al., 2008
Herpes zoster	8%	Galil et al., 1997
Stroke	8%	Andersen et al., 1995
Multiple sclerosis	28%	Österberg et al., 2005
Spinal cord injury	67%	Finnerup et al., 2001
Diabetes	26%	Abbott et al., 2011
HIV	50%	Schütz and Robinson-Papp, 2013
Cancer	~20%	Bennett et al., 2012

Table 3. Prevalence of neuropathic pain in the European population. Adapted from (Cousins *et al.*, 2010).

2.4.3 Clinical characteristics

2.4.3.1 Nociceptive and sensorial manifestations

Clinical manifestations of neuropathic pain are often discussed in terms of negative and positive symptoms. **Negative symptoms** indicate reduced impulse conduction in the neural tissues which are uncomfortable but not painful and include hypoesthesia, anesthesia, and hypoalgesia. By contrast, **positive symptoms** refer to painful sensations reflecting an abnormal level of excitability in the nervous system, which can be spontaneous or evoked by stimulation (Woolf, 2004). The classical stimulus-evoked pain suffered by neuropathic pain patients are **allodynia and hyperalgesia** (Scholz *et al.*, 2019). Depending on the nature of the stimulus, the resultant condition is

known as heat, cold or mechanical hyperalgesia. Definitions of both negative and positive symptoms are listed in **Table 4**.

Negative symptoms	
Hypoesthesia	Decreased sensitivity to stimulation (tactile or thermal)
Anesthesia	Lack of stimulation (tactile or thermal)
Hypoalgesia	Diminished pain response to a canonical painful stimulus
Positive symptoms	
Paraesthesia	An abnormal sensation
Dysesthesia	An unpleasant sensation
Paroxysmal pain	Intermittent spontaneous pain
Spontaneous pain	Continuous ongoing pain
Hyperalgesia	An increased response to a stimulus that is normally painful
Allodynia	Pain induced by non-noxious stimuli that normally do not activate the nociceptive system

Table 4. Definitions of common symptoms suggestive of neuropathic pain. Adapted from (Merskey H *et al.*, 1994).

2.4.3.2 Cognitive manifestations

Chronic pain patients usually present impairment of cognitive functions (prevalence ~11.4%) that can negatively influence their work productivity and daily life activities (**Fig. 15**) (Moriarty *et al.*, 2011). Some neuroanatomical substrates involved in cognition, such as the **mPFC** and the **hippocampus**, also participate in pain processing, suggesting a reciprocal modulation. It is suggested that chronic pain-induced cortical and hippocampal plasticity may underlie cognitive impairments through central sensitization (Woolf, 2011) and long-term potentiation-dependent mechanisms (Liu *et al.*, 2014). A wide range of cognitive tasks, including learning, memory, and executive functions,

have been reported altered in individuals suffering from neuropathic pain (Apkarian *et al.*, 2004; Muñoz and Esteve, 2005), as well as in preclinical animal (La Porta *et al.*, 2016; Martínez-Navarro *et al.*, 2019).

2.4.3.3 Emotional manifestations

Like other chronic pain conditions, neuropathic pain is frequently accompanied by emotional alterations with a ranging prevalence from 33% to 42% (Langley *et al.*, 2013). Clinical studies consistently report that patients with neuropathic pain are also afflicted with depression and anxiety disorder, a pattern that is also seen in animal models (Doan *et al.*, 2015; Colloca *et al.*, 2017; Descalzi *et al.*, 2017). Several animal studies using different neuropathic pain models showed increased **anxiety-like behaviors** in mice after peripheral nerve injury (La Porta *et al.*, 2016; Martínez Navarro, 2020; Wang *et al.*, 2021). The development of **depressive-like behavior** following a nerve injury was also reported in preclinical studies, as shown by increased behavioral despair in the forced swimming test and decreased sucrose preference in animals undergoing neuropathic pain (Gonçalves *et al.*, 2008; Leite-Almeida *et al.*, 2009; Wang *et al.*, 2011).

The causal relationship between persistent pain and emotional alterations is complex, since chronic pain leads to a negative affective state and, in turn, this negative state contributes to worsening pain perception (Bushnell *et al.*, 2013) (**Fig. 15**). Consistent evidence points to the **amygdala** as an important neural substrate of the interaction between pain and emotion. The existence of the nociceptive input via the spinoparabrachio-amygdaloid pathway, as well as clinical neuroimaging data showing amygdala activation in experimental and

clinical pain states, have implicated the amygdala as a critical node in emotional affective aspects of pain (Thompson and Neugebauer, 2019). Preclinical studies have also reported maladaptive plasticity and hyperactivity of the amygdala in animal models of neuropathic pain (Ikeda *et al.*, 2007; Gonçalves and Dickenson, 2012). Nevertheless, the amygdala is closely interconnected to limbic cortical areas, such as the PFC, contributing to the complexity and persistence of the emotional and executive consequences of chronic pain (Neugebauer *et al*, 2015).

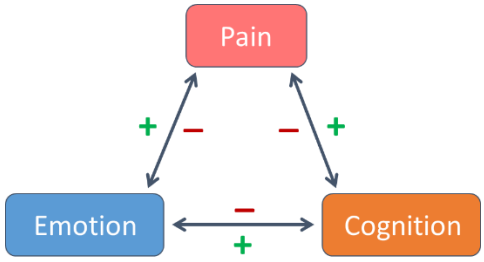

Figure 15. Feedback loops between pain, emotions, and cognition. Pain can have a negative effect on emotions and cognitive functions. Conversely, a negative emotional o cognitive state can lead to increased pain, whereas a positive state can reduce pain. Naturally, emotions and cognition can also reciprocally interact. Adapted from (Bushnell *et al.*, 2013).

2.4.3.4 Other manifestations

Besides emotional symptoms and cognitive deficits, other important comorbid manifestations of neuropathic pain that affect the patient's quality of life are sleep disturbances (prevalence ~37%-60%), and deficits in social behavior (Langley *et al.*, 2013). Therefore, it is of great importance the design of therapeutic strategies that not only tackle

nociceptive alterations, but also deal with the comorbid symptoms associated with persistent pain.

2.4.4 Adaptative changes leading to neuropathic pain after peripheral nerve injury

Different maladaptive changes in the peripheral and central nervous systems occur to develop and maintain neuropathic pain (Nickel *et al.*, 2012).

2.4.4.1 Peripheral sensitization

Sensitization of nociceptors

After a peripheral nerve lesion, damaged primary afferent neurons release pronociceptive mediators in the site of injury, including substance P, bradykinin, nitric oxide, and calcitonin gene-related peptide (Basbaum *et al.*, 2009). These **sensitizing agents** act on different receptors lowering their activation threshold and driving structural and functional changes in nociceptors, including collateral sprouting, alterations of ionic channels, ectopic and spontaneous discharges, abnormal nerve conduction, and nociceptor sensitization, which perpetuate pain experience (Scholz and Woolf, 2002).

Peripheral neuroimmune interactions

Besides neuronal modulation, resident non-neuronal cells also influence pain transmission in somatosensory areas, particularly under pathological neuropathic conditions. Indeed, damaged primary sensory neurons also release pronociceptive mediators that activate resident mast cells and macrophages, and vasoactive factors that promote the

infiltration of circulating immune cells to the site of injury (Scholz and Woolf, 2007). Subsequently, **activated immune cells** release several **pro-inflammatory mediators**, such as cytokines, chemokines, brain-derived neurotrophic factor (BDNF), pronociceptive substances (histamine, bradykinin), and reactive oxygen species, that activate and sensitize primary sensory neurons back, contributing to pain amplification (Austin and Moalem-Taylor, 2010; Ellis and Bennett, 2013). At the DRG level, activated satellite glial cells and infiltrated blood-derived immune cells also release pro-inflammatory factors that disrupt the homeostasis of the sensory neurons host in the DRG (Hu *et al.*, 2007; Morin *et al.*, 2007; Xie *et al.*, 2009).

Abnormal ectopic excitability of affected neurons

The sensitizing agents released after nerve injury also induce structural and functional alterations of ionic channels in primary afferent neurons. Clustering of Na^+ and Ca^{2+} channels at sites of ectopic impulse generation might be responsible for the lowering of the action-potential threshold and consequent spontaneous discharges of primary afferent sensory fibers (Misawa, 2012). After nerve injury, the concentration of messenger RNA for **voltage-gated Na^+ and Ca^{2+} channels** rises in the primary afferent neurons, not only at the injury site but also along the axon and the DRG (Luo *et al.*, 2001; J. Yang *et al.*, 2018).

2.4.4.2 Central sensitization

Multiple changes also occur in the spinal cord and supraspinal structures during the development of neuropathic pain. The peripheral

mechanisms described in the previous section usually lead to spontaneous painful sensations, whereas hyperalgesia and allodynia, are enhanced stimulus-evoked pain mainly related to both peripheral and central sensitization (Woolf and Mannion, 1999). Indeed, peripheral nerve injury also leads to hyperexcitability of the dorsal horn neurons in response to persistent noxious stimuli in the so-called **central sensitization** phenomenon (Latremoliere and Woolf, 2009).

Pronociceptive facilitation at the spinal dorsal horn

Pathologically hyperexcited C-fibers further sensitize spinal dorsal horn neurons by releasing glutamate and substance P into the spinal cord (Basbaum *et al.*, 2009). **Glutamate** is the major excitatory neurotransmitter of the pain system and exerts its effect via α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid receptors (AMPA), kainate, N-methyl-D-aspartate (NMDA), and particularly metabotropic glutamate receptors (mGluR). Under pathological conditions, such as neuropathic pain, ongoing nociceptive input triggers excessive glutamate release that induces the activation of mGluRs (J. W. Wang *et al.*, 2012). Notably, metabotropic glutamate receptors of group I, mGluR1 and **mGluR5**, elicit adaptive changes, especially through activating MAPK pathways, that contribute to the hyperexcitability of spinal dorsal horn projecting neurons (Vincent *et al.*, 2016; Xie *et al.*, 2017). By contrast, calcitonin gene-related peptide and substance P released from C fiber terminals contribute to the central sensitization at the spinal cord level by promoting the disinhibition of spinal NMDA receptors and the ensuing Ca^{2+}-dependent neurochemical changes in the postsynaptic neurons (D'Mello and Dickenson, 2008). As a result,

second-order neurons at the spinal cord level become hyperactivated and show enhanced stimulus-evoked pain responses, such as allodynia and hyperalgesia (Nickel *et al.*, 2012).

Neuroimmune interactions

Glial and immune cells also play an important role in the pathogenesis and maintenance of neuropathic pain, as well as the proinflammatory mediators released after nerve injury (Scholz and Woolf, 2007). The release of nociceptive mediators from damaged primary afferent neurons triggers glial reactivity at central levels (Ren and Dubner, 2010). It has been reported a strong activation of spinal **microglia** in several models of chronic pain (Tsuda *et al.*, 2005; Negrete *et al.*, 2017; Martínez-Navarro *et al.*, 2019). Spinal **astrocytes** also become activated after tissue damage, but with a slower onset than microglia. This delayed activation is suggested to play a role in the maintenance of neuropathic pain (Ji *et al.*, 2013). In turn, both reactive microglia and astrocytes release the pro-inflammatory factors previously mentioned in section 2.4.4.1 promoting increased neuronal excitability in the dorsal horn of the spinal cord and prolonging synaptic pain transmission. Finally, infiltration of other immune cells, including **mast cells**, **macrophages,** and **lymphocytes,** has also been demonstrated in models of peripheral neuropathic pain (Hu *et al.*, 2007; Cao and DeLeo, 2008; Baral *et al.*, 2019). Particularly, cells of the adaptative immune system, namely T and B cells, contribute to the regulation of pain transmission during chronic pain states (Sorge *et al.*, 2015; Cabañero *et al.*, 2020). Therefore, immune cells not only contribute to peripheral sensitization of nociceptors, but also interact with glial cells in the

spinal cord to increase the excitability of the dorsal horn neurons, thus contributing to the maintenance of neuropathic pain.

Disinhibition of nociception at the spinal inhibitory network

Changes in **descending inhibitory pathways** are also important in the central sensitization produced during neuropathic pain (Woolf and Mannion, 1999). These projecting neurons receive strong inhibitory input from descending serotonergic, noradrenergic, and dopaminergic pathways originating from the PAG, locus coeruleus, and the RVM (Nickel *et al.*, 2012). In line with this, it has been shown a reduced activity and efficacy of the descending inhibitory pathways in mice undergoing neuropathic pain (Zimmermann, 2001). In addition, **inhibitory interneurons** within the dorsal horn constitute local circuits that also play a role in inhibiting nociceptive transmission (Nickel *et al.*, 2012). Thus, selective loss of inhibitory GABAergic interneurons of the dorsal horn is observed after peripheral nerve injury in rodents (Moore *et al.*, 2002), which further leads to central sensitization.

Supraspinal reorganization processes

Most animal experiments investigating the mechanisms involved in central sensitization have been focused on the dorsal horn of the spinal cord. However, adaptive changes in supraspinal structures have also been reported including somatosensory cortices and thalamus (McCarberg and Peppin, 2019). Cortical reorganization processes were revealed in rats following a peripheral nerve injury (Brüggemann *et al.*, 2001) and in patients with phantom limb pain (Flor *et al.*, 1995) and central pain syndrome (Maihöfner, 2014). Furthermore,

hyperexcitability of the ACC has been described in different mouse models of neuropathic pain, driving to aversive learning associated with chronic pain states (Meda *et al.*, 2019). Aside from cortical alterations, neuroplastic changes in the thalamus and brain stem nuclei have been described during ongoing pathological pain using positron emission tomography and functional magnetic resonance imaging studies. During chronic pain, the **thalamus** shows a decreased baseline activity or stimulus-related activity after spinal cord injury (Garcia-Larrea and Peyron, 2013; Gustin *et al.*, 2014)**,** suggesting that this area is undergoing adaptive changes. On the other hand, the **amygdala** and **mPFC** of neuropathic pain patients exhibit increased activation (Apkarian *et al.*, 2005), implying that persistent pain alters the cognitive and emotional responses of individuals. Physiological and biochemical changes in **PAG and RVM** under neuropathic pain conditions have been also reported in humans patients (Vanegas and Schaible, 2004; Seifert and Maihöfner, 2009).

Overall, the development of neuropathic pain involves not only neuronal alterations, but also immune and glial cells that participate in the modulation of pain transmission at the peripheral and central levels.

2.4.5 Mouse models of neuropathic pain

Animal models of neuropathic pain have been used to study the mechanisms underlying the pathophysiology of this disease and to design novel therapeutic strategies to obtain effective compounds for clinical use (Bridges *et al.*, 2001).

The experimental animal models of neuropathic pain developed in the last decades include both central and peripheral nervous system injuries. **Table 5** summarizes the main neuropathic pain experimental models that can be classified into four categories: nerve injury models, drug-induced neuropathic pain, disease-induced neuropathy, and miscellaneous ones. Among the available models, peripheral nerve injuries caused by a mechanical method are frequently used due to better accessibility to induce the damage. The three most used peripheral models in rodents are the chronic constriction injury (CCI) of the sciatic nerve (Austin *et al.*, 2012), the partial sciatic nerve ligation (PSNL) (Seltzer *et al.*, 1990; Malmberg and Basbaum, 1998), and the spinal nerve ligation (SNL) (Chung *et al.*, 2004). A schematic view of the site of injury of the most used peripheral nerve injury models is depicted in **Figure 16.**

These models have been proved to reproduce accurately the typical sensory alterations associated with neuropathic pain (allodynia and hyperalgesia) (Bridges *et al.*, 2001). However, animal models of neuropathic pain give few clues about the clinical side effects of the drugs, and this has been one of the most important limitations of neuropathic pain treatments (Kumar *et al.*, 2018).

1. Nerve injury
Central pain
• ***Spinal cord injury***
Excitotoxins, contusion, photochemical model
• ***Spinal hemisection***
• ***Thalamic syndrome***
Peripheral pain
• ***Complete lesion***
Sciatic nerve transection (neuroma model)
Brachial plexus avulsion
• ***Partial lesion***
Sciatic nerve chronic constriction injury (CCI)
Partial sciatic nerve ligation (PSNL)
Spinal nerve ligation (SNL)
Cuffing of the sciatic nerve
Caudal trunk resection
Spared nerve injury (SNI)
Sciatic cryoneurolysis
Sciatic inflammatory neuritis
Trigeminal neuralgia
2. Drug-induced neuropathy
• ***Anti-cancer agents***
Vincristine
Cisplatin
Taxanes
• ***Anti-retroviral drugs***
Didanosine
Zalcitabine
Stavudine
3. Disease-induced neuropathy
Diabetes (streptozotocin-induced peripheral diabetic neuropathy)
Cancer pain model
HIV-induced
Post herpetic neuralgia model
4. Miscellaneous
Ethanol consumption/withdrawal-induced neuropathy
Pyridoxine (vitamin B6)-induced neuropathy
Inherited-induced neuropathies (Charcot-Marie-Tooth)
Uremic peripheral neuropathy (end-stage kidney disease)

Table 5. Classification of main neuropathic pain models. Adapted from (Kumar *et al.*, 2018).

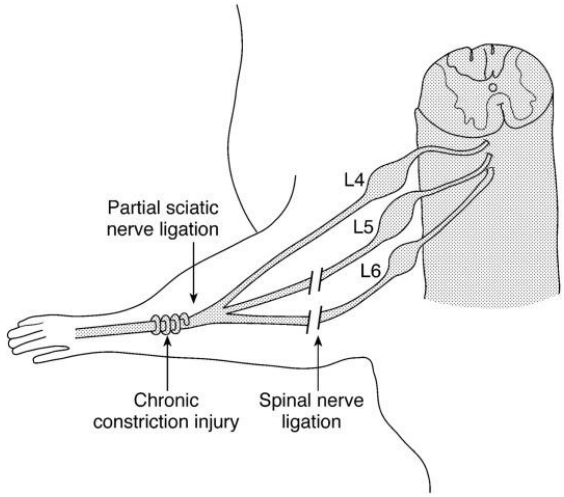

Figure 16. Schematic drawing of the most used peripheral nerve injury models. Chronic constriction injury (CCI) comprises loose ligations of the sciatic nerve trunk. The partial sciatic nerve ligation (PSNL) is tight ligation of 33-50% of the sciatic nerve trunk, whereas spared nerve injury (SNL) consists of a tight ligation and a transection of the L5 and L6 spinal nerves. Extracted from (Bridges *et al.*, 2001).

2.4.6 Therapeutic approaches for neuropathic pain

Nowadays, there is not a fully effective treatment to palliate all the symptoms of neuropathic pain despite the many approaches available. Treatments are often directed to diminish pain and help patients to cope with their symptoms rather than suppress pain (Woolf and Mannion, 1999). Besides being undertreated, neuropathic pain is often underdiagnosed since recent epidemiological surveys have shown that many patients with neuropathic pain do not receive appropriate treatment (Attal *et al.*, 2011; Torrance *et al.*, 2013). In addition, the inter-individual variability of neuropathic pain symptoms and its emotional and cognitive comorbidities complicate the diagnostic

process. Therefore, the current management of neuropathic pain comprises the integration of multidisciplinary pharmacological and non-pharmacological therapies.

Regarding the **pharmacological therapies**, non-opioid medications such as tricyclic antidepressants (TCAs), dual serotonin norepinephrine reuptake inhibitors (SNIRs), gabapentanoids and topicals, are recommended as **first-line therapy** (**Fig. 17**). Besides their analgesic effects, TCAs and SNIRs are considered first-line therapy due to their antidepressant properties that may be also beneficial to the emotional comorbidities associated with chronic neuropathic pain (Bates *et al.*, 2019). The main analgesic action of these drugs is to enhance the descending inhibitory pathways by blocking the reuptake of serotonin and noradrenaline neurotransmitters (Baron *et al.*, 2010; Kremer *et al.*, 2016). Gabapentanoids, such as gabapentin and pregabalin, are voltage-gated Ca^{2+} channel antagonists that ameliorate neuropathic pain by inhibiting neuronal transmission at the level of the spinal dorsal horn, reducing excitability of afferent neurons, and facilitating descending inhibitory control of pain (Patel and Dickenson, 2016). Finally, topicals such as capsaicin alleviate neuropathic pain by activating and desensitizing TRPV1 receptors in the nociceptors, whereas lidocaine blocks Na^{+} channels to subsequently reduce peripheral sensitization (Attal *et al.*, 2010; Baron *et al.*, 2010). Both medications are topically administered for focal and peripheral neuropathic pain (Bates *et al.*, 2019).

Combination therapy of first-line medications and tramadol are recommended as **second-line therapy** (**Fig. 17**). Tramadol has multiple

mechanisms of action, but primarily acts as a weak μ-opioid agonist and inhibitor of serotonin and norepinephrine reuptake that is effective in patients with a variety of neuropathic pain conditions (Bates *et al.*, 2019).

For patients who do not tolerate or do not achieve sufficient pain relief from first- or second-line therapy, the use of serotonin-specific reuptake inhibitors (SSRIs), anticonvulsants, such as carbamazepine, topiramate, and sodium valproate, and NMDA antagonists is recommended as **third-line therapy** and neurostimulation as a **fourth-line treatment** before commencing low-dose of classical opioids (**Fig. 17**).

The effectiveness of opioids, including oxycodone, morphine, methadone, and levorphanol extends from nociceptive to neuropathic pain states (Corder *et al.*, 2018). However, current guidelines suggest that opioids should be firmly considered **fifth-line therapy** (**Fig. 17**), after a trial of neurostimulation has been attempted (Bates *et al.*, 2019). The analgesic effects of opioid agonists in neuropathic pain are mediated by reducing the excitability of afferent neurons, modulating pain integration in supraspinal areas, and increasing descending inhibition of pain transmission into the dorsal horn (Nadal *et al.*, 2013). However, opioid compounds have the potential to develop tolerance and dependence, as well as important side effects, such as nausea, vomiting, respiratory depression, constipation, confusion, and sedation, that can limit their use in patients (Finnerup *et al.*, 2015). For this reason, the clinical use of opioids to manage neuropathic pain

states is restricted to fifth-line therapy due to their dependence/side effect profile (Bates *et al.*, 2019).

Finally, targeted drug delivery is the last-line treatment, **sixth-line therapy**, for patients that after appropriate conservative, pharmacological, and interventional management has failed to achieve an acceptable quality of life for the patient (Bates *et al.*, 2019) (**Fig. 17**).

Nevertheless, pain is more than just an unpleasant sensation. It can encompass emotional and cognitive alterations, as well as other important comorbid manifestations, such as functional, sleep, mood, and social disturbances, that drive patient's quality of life (Bates *et al.*, 2019). Therefore, **non-pharmacological treatments** are also a key component for the management of neuropathic pain to address issues such as depression, anxiety, sleep disturbance, or social interactions. Among them, it is worth mentioning the benefits of exercise, massage, and supportive psychotherapy (Evans *et al.*, 2003; Bates *et al.*, 2019). However, there are cases in which the only effective solution to treat neuropathic pain is surgery, such as traumatic peripheral nerve injury, lumbar disk hernia, or neuromas (Moulin *et al.*, 2007).

The search for more effective and safe drugs is still imperative to improve the quality of life of neuropathic pain patients. No one drug works for all patients, and pain relief is usually partial (Sindrup and Jensen, 1999; Collins and Chessell, 2005). Not surprisingly, 45% of patients with neuropathic pain utilize two or more medications for their pain condition (Bates *et al.*, 2019). Furthermore, important side effects often limit the ability to achieve adequate pain control with a single agent, leading either to discontinuation of the treatment or to

use of more than one drug to optimize pain control (Namaka *et al.*, 2009). For that purpose, one objective of this thesis was to study the therapeutic potential of CB2R in a mouse model of neuropathic pain to alleviate the nociceptive and emotional manifestations typically associated with this condition.

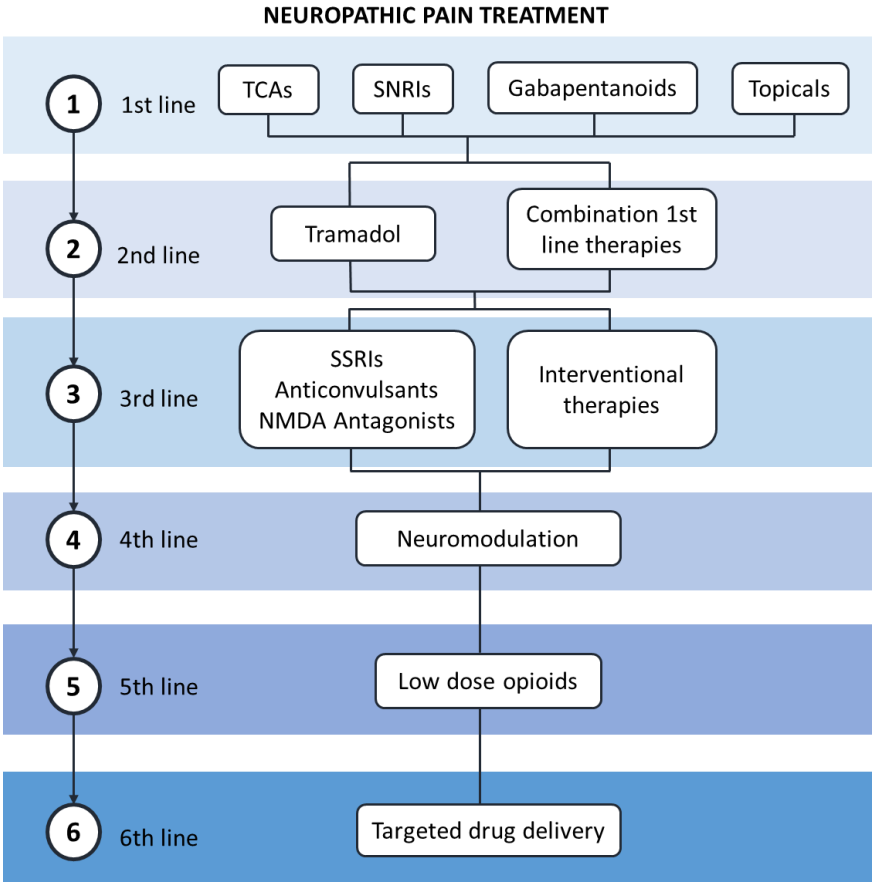

Figure 17. Comprehensive algorithm for the management of neuropathic pain. TCAs: Tricyclic antidepressants, SNRIs: Serotonin norepinephrine reuptake inhibitors, NP: neuropathic pain, SSRIs: Selective serotonin reuptake inhibitors, NMDA: N-methyl-D-aspartate. Adapted from (Bates *et al.*, 2019).

2.5 The endocannabinoid system in neuropathic pain

Despite not being approved for the treatment of neuropathic pain (Bates *et al.*, 2019), cannabinoids can modulate pain transmission by mainly reducing neurotransmitter release of different systems (glutamate, GABA, DA, among others) at peripheral and central levels (Kano *et al.*, 2009). Due to their anatomical widespread distribution in the CNS, cannabinoids also affect other components of pain perception, including emotional and cognitive comorbidities, acting in cortical and limbic areas (Nadal *et al.*, 2013). However, the development of cannabinoid agonists as analgesics has been hampered due to psychotropic side effects, and the prejudice generated by the recreational use of marijuana.

Recent evidence has shown the potential analgesic effect of cannabinoid agonists in different neuropathic pain animal models and human trials (Maldonado *et al.*, 2016; Shang and Tang, 2017). Indeed, some clinical trials with Sativex® spray (containing 50/50 THC and cannabidiol) have shown symptomatic relief in different chronic neuropathic pain syndromes (Serpell *et al.*, 2014; Hoggart *et al.*, 2015). Regarding the endogenous cannabinoid system, both CB1R and CB2R activation produce antinociception in neuropathic pain models (Davis, 2014). Several preclinical studies have reported that natural and synthetic cannabinoids are effective in the attenuation of neuropathic pain through **CB1R** mechanisms (Ji and Neugebauer, 2014; Hossain *et al.*, 2020). Spinal and supraspinal CB1R are upregulated in many murine models following nerve injury, and this may account for the efficacy of CB1R agonists in neuropathic pain (Banister *et al.*, 2019). The analgesic

action of CB1R is predominantly mediated by the central inhibition of painful stimuli in the dorsal horn of the spinal cord (Pacher *et al.*, 2006) and by stimulating the descending inhibitory pathway from PAG and RVM structures (Palazzo *et al.*, 2010), with a lower peripheral involvement. Nevertheless, CB1 activation leads to important psychoactive, motor, and cognitive effects, which represent one of the major limitations of using CB1R agonists for chronic pain treatment (Davis, 2014). Alternative approaches have been developed to overcome this problem by targeting **CB2R** that is predominantly expressed in peripheral immune cells (Svízenská *et al.*, 2008). As it has been described in section 2.4.4.2, glial and immune cells also play an important role in the pathogenesis and maintenance of neuropathic pain. Various studies have reported that reactive microglia interact with neurons and contribute to the development of neuropathic pain (Tsuda *et al.*, 2005; Negrete *et al.*, 2017; Martínez-Navarro *et al.*, 2019). CB2R are also present in microglial cells which were observed to be upregulated following inflammation or nerve injury (Duffy *et al.*, 2021). Thus, CB2R activation with agonists drugs is efficient to attenuate the manifestations of neuropathic pain after nerve injury (Gutierrez *et al.*, 2011; Niu *et al.*, 2017; Cabañero *et al.*, 2020). In accordance, genetic studies have demonstrated that CB2KO mice show a reduction of neuropathic pain manifestations, whereas CB2R overexpression worsens these symptoms (Racz *et al.*, 2008a, 2008b). A similar study showed that mice lacking CB2R on microglia develop mirror-image allodynia similar to constitutive CB2KO mice after nerve ligation, a phenotype that was not observed after the deletion of CB2R from neurons (Nent et al., 2019).

CB2R in microglia have been more extensively researched than CB1R due to their neuroprotective actions and lack of psychotropic effects commonly associated with CB1 agonists (Bie *et al.*, 2018). Therefore, CB2R could be a potential therapeutic target for neuropathic pain treatment avoiding the risk of centrally-mediated side effects.

3 Neurobiology of food intake

Eating, drinking, or sex are natural stimuli with intrinsically reinforcing properties since all can activate the reward system (Volkow *et al.*, 2017). Notably, the regulation of food intake comprises tight relationships between homeostatic (feeding to satisfy biological needs) and non-homeostatic hedonic factors (eating for pleasure), which balance ensures the initiation and maintenance of eating behavior (Onaolapo and Onaolapo, 2018). Both regulatory circuits are activated during feeding situations and must work together to maintain the nutritional status of the individual adapted to the environment. However, in our developed society, where food shortage is not an issue, reward-related signals can prevail over homeostatic signals potentiating excessive food intake above the body's energy requirement and leading to eating disorders (Pandit *et al.*, 2011; Caron and Richard, 2017).

3.1 Food intake control

3.1.1 Homeostatic regulation of food intake

The homeostatic control or energy balance is mediated by the biological need to maintain body weight and metabolic functions. It requires reciprocal communication between peripheral organs that act as the body's energy sensors for nutrient status, and the brain which integrates these inputs generating an intake outcome considering the external environmental availability of food (Lutter and Nestler, 2009). Several organs in the body, including the gastrointestinal tract, the

adipose tissue, and the central nervous system, act together to maintain an appropriate energy balance (Abdalla, 2017).

Central regulation

The central regulation of homeostatic feeding is an organized circuit where inputs from the periphery are integrated mainly by the hypothalamus, and descending outputs are sent to peripheral tissues through the vagal and spinal nerves (Kalon *et al.*, 2016).

The **hypothalamus** is the main relay center of afferent signals from peripheral organs that orchestrate homeostatic feeding behavior (Williams and Elmquist, 2012; Kalon *et al.*, 2016). This brain area receives sensory information about stomach mechanical distension, chemical signals of satiety from nutrients in the blood, hormonal signals from the gastrointestinal tract and adipose tissue, and central signals from the cerebral cortex (taste, smell, and sight food). The hypothalamus processes all this information and sends efferent signals to control food intake. Several interconnected nuclei relevant for appetite control conformed the hypothalamus, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial nucleus, the ventromedial nucleus (VMH), and the lateral hypothalamic area (LH) (Williams and Elmquist, 2012) (**Fig. 18**).

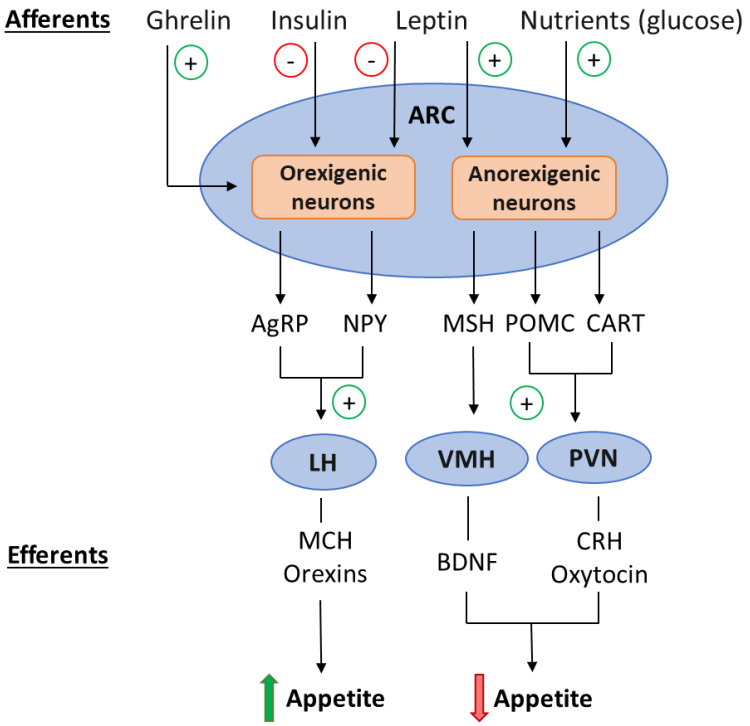

Figure 18. A simplified scheme of regulation of energy homeostasis by hypothalamus nuclei. Energy homeostasis is an organized circuit where afferent signals coming from the periphery are integrated into specific hypothalamic nuclei that processes all this information and sends efferent signals to control food intake. ARC: arcuate nucleus, LH: lateral hypothalamus, VMH: ventromedial hypothalamus, PVN: paraventricular nucleus, AgRP: agouti-related peptide, NPY: neuropeptide Y, MSH: alpha-melatonin stimulating hormone, POMC: proopiomelanocortin, CART: cocaine-amphetamine-related transcript, MCH: melanin-concentrating hormone, BDNF: brain-derived neurotrophic factor, CRH: corticotropin-releasing hormone. Adapted from (Haliloglu and Bereket, 2015).

The **ARC** is targeted by several satiety hormones released from the gastrointestinal tract and adipose tissue to regulate food intake. It contains a mixed population of orexigenic (anabolic) and anorexigenic (catabolic) neurons that project to other hypothalamic areas involved

in appetite control (Abdalla, 2017). Orexigenic neurons express neuropeptide Y (NPY) and agouti-related protein (AgRP) and are inhibited by insulin and leptin. In contrast, anorexigenic neurons are stimulated by leptin and nutrients and co-express pro-opiomelanocortin (POMC), the precursor of melanocyte peptides stimulating hormone, α-, β-, γ- MSH, and cocaine- and amphetamine-regulated transcript (CART). The NPY/AgRP positive neurons stimulate food intake projecting to the LH, whereas the POMC/CART positive neurons project to the **PVN** that suppresses feeding by secreting CRH and oxytocin (Haliloglu and Bereket, 2015). The **LH** works as a hunger center, promoting feeding behavior (Rossi *et al.*, 2019). Neurons from this area express two peptides that drive food-seeking: melanin-concentrating hormone and orexins (Li *et al.*, 2014). In contrast, the **VHM** performs the role of a satiety center (Mishra *et al.*, 2017). Specifically, the VMH receives POMC neuronal projections from the arcuate nucleus that activate BDNF neurons to decrease food intake (Haliloglu and Bereket, 2015; Abdalla, 2017) (**Fig. 18**).

Peripheral regulation

Peripheral signals and circulating hormones are released from the gastrointestinal tract, pancreas, liver, muscles, and adipose tissue to provide information to the CNS about the body's energy status (Mishra *et al.*, 2017). Many of them are peptide neurotransmitters which receptors are in the hypothalamic nuclei involved in feeding and satiety behaviors. These neurotransmitters are commonly classified as **orexigenic substances** that stimulate food intake or **anorexigenic substances** that inhibit food intake (**Table 6**).

Orexigenic substances	Anorexigenic substances
Neuropeptide Y	α-MSH
AgRP	Leptin
Melatonin concentrating hormone	Serotonin
Orexin A	CRH
Orexin B	Norepinephrine
Endorphins	Insulin
Ghrelin	Glucagon-like peptide
Cortisol	Cholecystokinin
	CART
	Peptide YY

Table 6. orexigenic and anorexigenic substances release from peripheral organs. Adapted from (Mishra *et al.*, 2017).

The main hormones affecting food intake include ghrelin, insulin, and leptin, secreted by the stomach, pancreas, and adipose tissue, respectively (Abdalla, 2017). Briefly:

- **Ghrelin** is an orexigenic peptide secreted mainly from the stomach and the duodenum. It is considered the "hunger" hormone because it produces meal inhibition and it has pre-prandial elevation, whereas these levels fall after food intake. Its main role is stimulating food intake and energy storage to compensate for a negative energy imbalance. In accordance, ghrelin circulating levels are high before meals and low after ingestion of nutrients (Date *et al.*, 2002; Drazen *et al.*, 2006). In the CNS, ghrelin receptors are found in NPY/AgRP hypothalamic neurons of the ARC and the reward system. Thus, ghrelin influences coordinately homeostatic and hedonic mechanisms of food intake (Skibicka *et al.*, 2011).

- **Insulin** is a pancreatic peptide hormone synthesized in the β cells of the pancreatic islets of Langerhans. Insulin is the primary anabolic hormone of the body. During meals, insulin is released in the blood and targets the liver to reduce glucose production and stimulate glucose uptake by peripheral tissues. Apart from its role in glucose metabolism, insulin also circulates in the bloodstream in proportion to white fat deposits serving as a sensor of body fat content to the hypothalamus (Gerozissis, 2004). Insulin receptors are found in the ARC of the hypothalamus together with orexigenic NPY/AgRP and anorexigenic POMC positive neurons. Insulin achieves its role in reducing feeding by inhibiting NPY/AgRP peptide production while enhancing POMC expression (Dodd and Tiganis, 2017).
- **Leptin** is a hormone secreted mainly by the adipose tissue that induces satiety, inhibits food intake, and increases catabolism to reduce excessive energy stores (Lutter and Nestler, 2009). The levels of leptin in the blood positively correlated with the amount of body fat. In fed conditions, blood leptin increases, whereas it falls in deprivation (Sitar-Taut *et al.*, 2020). Leptin produces its anorexigenic effect in the ARC, inhibiting NPY/AgRP neurons and activating POMC/CART neurons, leading to reduced food intake and increased energy waste (Abdalla, 2017).

In summary, the control of food intake is performed by hypothalamic neurons of the ARC which secret orexigenic and anorexigenic neuropeptides under stimulation of ghrelin, insulin, and leptin. Thus, energy homeostasis depends on high nervous centers to integrate and

generate an adequate response to these peripheral hormonal signals. Dysregulation of the synthesis of these hormones can lead to an increased caloric intake, which, in turn, is a significant risk factor for obesity and its comorbidities (Benite-Ribeiro *et al.*, 2021). Indeed, obesity is accompanied by alterations of ghrelin, insulin, and leptin serum levels (Sitar-Taut *et al.*, 2020; Skuratovskaia *et al.*, 2021).

3.1.2 Hedonic regulation of food intake

The feeding behavior is not only influenced by internal homeostatic signals but also by non-homeostatic factors. Food palatability represents an important component involved in regulating food intake even in periods of energy abundance (Kure Liu *et al.*, 2019). Furthermore, environmental cues (Agarwal *et al.*, 2021) and emotional states (Benzerouk *et al.*, 2018) are also implicated in the decision to eat.

The **hypothalamus**, especially the LH, is highly connected to cortical and subcortical brain areas involved in the hedonic pleasure, emotion, and memory of feeding behavior. This corticolimbic appetite network comprises the **executive system** and the **reward system**, both responsible for the hedonic regulation of food intake (Volkow *et al.*, 2012; Kalon *et al.*, 2016).

The cortical **executive system** integrates gustatory, olfactory, visual, and somatosensory inputs from the thalamus, such as smell, sight, and taste, and is responsible for the conscious and voluntary decision of eating (**Fig. 19**). Within the cortex, the insular cortex and the orbitofrontal cortex (OFC) have a notable role in the processing of

palatability (Caron and Richard, 2017). Furthermore, the PFC is densely interconnected with other cortical brain regions, such as the "**default mode network**" (DMN). Both substance use disorders and obesity are associated with impairments in the executive control network and the DMN, suggesting that they may contribute to their etiology (Goldstein and Volkow, 2011; Zhang and Volkow, 2019). The DMN is a network that includes the mPFC, posterior cingulate cortex, cuneus/precuneus, medial temporal lobe, and inferior parietal cortices. Importantly, several studies have reported alterations within the areas and connectivity of the DMN in obese subjects compared to healthy-weight individuals (Agarwal *et al.*, 2021).

By contrast, the subcortical **reward system** is responsible for the hedonic component of feeding. In this circuit, two interconnected limbic areas are involved, the **VTA** and the **NAc**, and the main neurotransmitter implicated is DA (Solinas *et al.*, 2018). **DA** reward pathway from VTA to NAc is responsible for the motivation towards food consumption and the pleasure associated with its consumption (Wang *et al.*, 2009). In addition, there is a distinction between "wanting", more associated with DA, and "liking" more associated with cannabinoid and opioid modulation (Berridge, 2009). **"Liking"** refers to the palatability or hedonic value associated with food, whereas **"wanting"** is considered the desire that stimulates goal-directed behavior to obtain the food that is often regarded as motivation (Koob, 2015). These two components are separate mechanisms that work together to modulate eating behavior (Zheng and Berthoud, 2007).

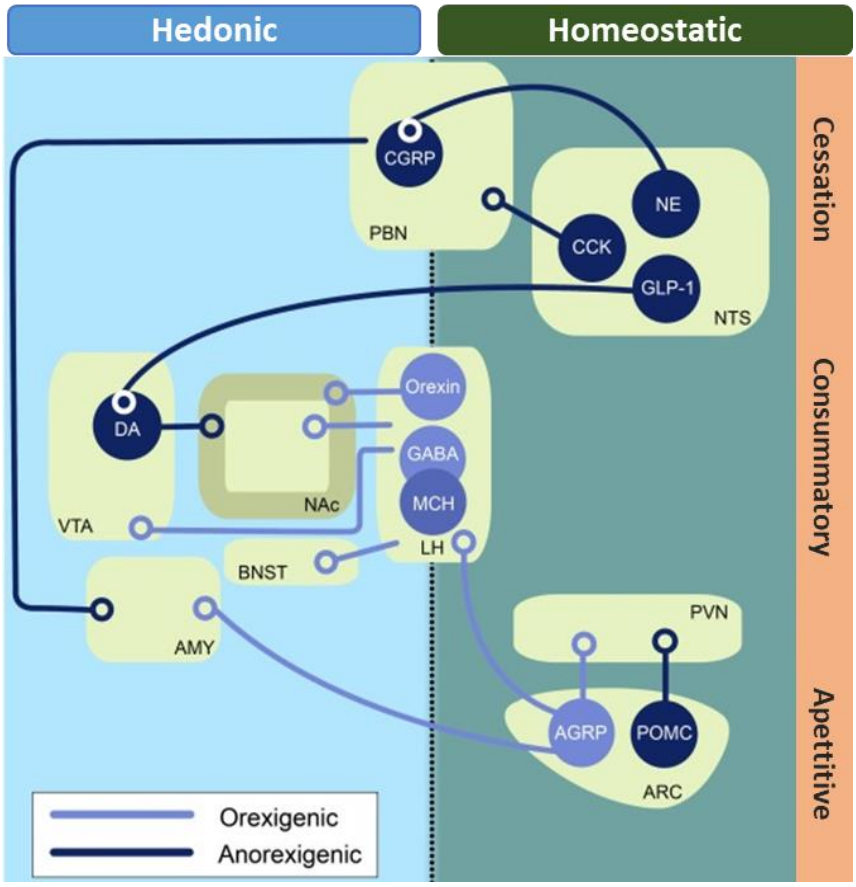

Figure 19. Interaction of the homeostatic and hedonic system in the control of appetitive, consummatory, and cessation stages of feeding. Schematic diagram showing recruitment of feeding nodes involved in the control of food intake and regulation of energy balance. Light blue lines denote typically orexigenic pathways, dark blue typically anorexigenic. AGRP: agouti-related peptide, AMY: amygdala, BNST: bed nucleus of the stria terminalis, ARC: arcuate nucleus of the hypothalamus, CCK: cholecystokinin, CGRP: calcitonin gene-related peptide, DA: dopamine, GLP-1: Glucagon-like peptide 1, LH: lateral hypothalamus, MCH: melanin-concentrating hormone, NAc: nucleus accumbens, NE: norepinephrine, NTS: nucleus of the solitary tract, PBN: parabrachial nucleus, POMC: proopiomelanocortin, PVN: paraventricular nucleus of the hypothalamus, VTA: ventral tegmental area. Adapted from (Massa and Correa, 2020).

Furthermore, **metabolic signaling** can also directly act on the brain reward circuit since both NAc and VTA areas express peptides and hormonal receptors for ghrelin, leptin, insulin, glucose, and glucagon-like peptide-1, among others. Indeed, in the VTA, leptin inhibits DA neuronal activity and decreases food intake (Domingos *et al.*, 2011), whereas ghrelin increases VTA DA activity and promotes food intake (**Fig. 19**) (Skibicka *et al.*, 2011). Therefore, peripheral hormones and neuropeptides directly modulate the DA reward system indicating important crosstalk between the homeostatic and hedonic systems (Volkow *et al.*, 2013).

Besides the NAc, other mesocorticolimbic structures have been shown to influence eating behavior. In particular, the projections from the amygdala and hippocampus to the hypothalamus play an important role in emotional and cognitive satiation signals (**Fig. 19**) (Berthoud, 2006). The **amygdala** oversees the motivational value of food consumption since a reciprocal connection exists between the basolateral amygdala and the forebrain that potentiates cue-related feeding (Kalon *et al.*, 2016; Volkow *et al.*, 2019). Moreover, projections between the central nucleus of the amygdala and the NAc are involved in opioid-mediated eating ("liking" component) (Kim *et al.*, 2004). The **hippocampus** is also involved in feeding behavior through its processing of memories (Volkow *et al.*, 2012). Indeed, rats with impaired hippocampal function showed a significant increase in hedonic feeding (Davidson *et al.*, 2007), while brain-imaging studies in humans have reported activation of this area in food craving, hunger, trying new food, and in response to food-conditioned cues (Agarwal *et*

al., 2021). In addition, the hippocampus also integrates memory-based and reward information arising from the **olfactory bulb** that determines behavioral responses to odors in the environment, particularly in the context of food. Indeed, many regions in the olfactory network are negatively affected by obesity and alcohol use disorders, in which smells acquire strong reinforcing value (Agarwal *et al.*, 2021). In agreement, a bidirectional connection exists between the olfactory tubercle and NAc with the hypothalamus and VTA (Koob and Volkow, 2010).

3.2 The brain reward system

Natural rewards such as food, water, and sex trigger rewarding effects through activating the **brain reward system**. This brain circuitry is mainly comprised of dopaminergic neurons from the VTA that project DA to cortical and limbic areas, forming the mesocortical and mesolimbic pathways, respectively. Within the **mesolimbic pathway**, dopaminergic neurons emerging from the VTA release DA into the NAc (ventral striatum), a key area involved in motivation and incentive salience. According to the behavioral reactions elicited, the motivational value of environmental stimuli can be rewarding or aversive. Regarding the **positive reinforcement**, exposition to appetitive stimuli activates the VTA-NAc pathway promoting goal-directed behaviors to obtain the reward (Koob, 2015; Koob and Volkow, 2016). Rewards induce subjective feelings of pleasure and positive emotional states. All known drugs of abuse increase extracellular levels of DA in the NAc (Manzanares *et al.*, 2018). In contrast, aversive stimuli act in the same circuit but with opposite

effects, promoting avoidance behaviors and contributing to **negative reinforcement** (Koob, 2017). Exposition to aversive conditions such as chronic pain, unavoidable shock, over or under-eating, and withdrawal from addictive drugs produces decreased DA levels in the NAc, contributing to aversive emotional states. These aversive states induced by negative reinforcement mechanisms are primarily mediated by the extended amygdala circuit (Parsons and Hurd, 2015) (**Fig. 20**).

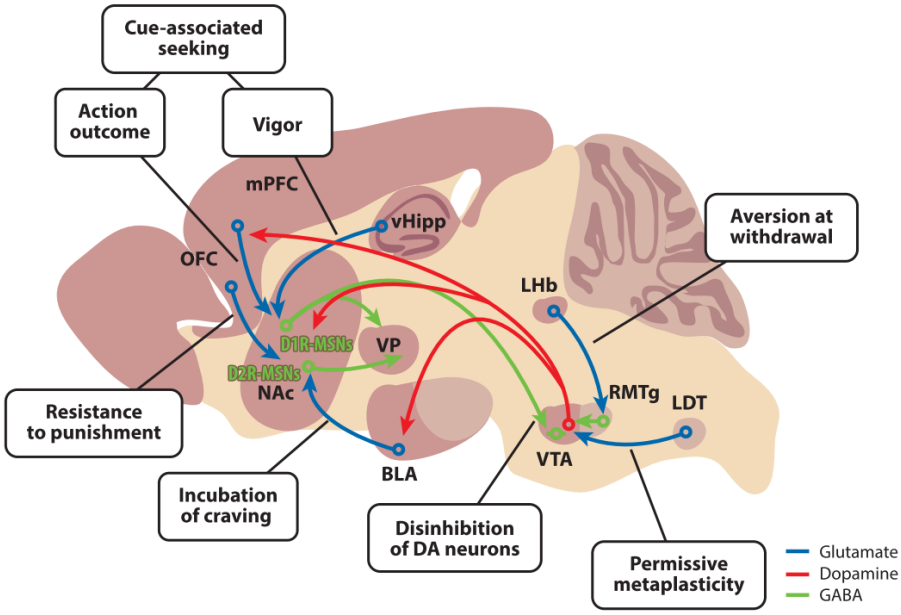

Figure 20. Reward system connectivity. Major connections undergoing reward-seeking behaviors in a sagittal section of the brain. Red indicates dopamine, blue indicates glutamate, and green indicates GABA. BLA: basolateral amygdala, D1R: dopamine D1 receptor, D2R: dopamine D2 receptor, LDT: laterodorsal tegmentum, LHb: lateral habenula, mPFC: medial prefrontal cortex, MSN: medium spiny neuron, NAc: nucleus accumbens, OFC: orbitofrontal cortex, RMTg: rostromedial tegmentum, vHipp: ventral hippocampus, VP: ventral pallidum, VTA: ventral tegmental area. Extracted from (Lüscher, 2016).

The following sections explain the cytoarchitecture and the principal connections of the main areas of the brain reward system for a better understanding of this complex circuitry.

3.2.1 Ventral tegmental area

The VTA processes both natural (eating, drinking, coupling) and non-natural (drugs, gambling) environmental stimuli creating a rewarding effect by DA release into de NAc. The VTA contains a heterogeneous mixture of dopaminergic projecting neurons (~60%), GABAergic projection neurons and interneurons (~30-35%), and glutamatergic neurons (2-3%) (Margolis *et al.*, 2012). VTA dopaminergic neurons mainly express the enzyme tyrosine hydroxylase and release DA. Nevertheless, some dopaminergic neurons co-express the GABA synthesizing enzyme glutamic acid decarboxylase 65Kd isoform (GAD65) or the vesicular glutamate transporter 2, leading to the release of GABA and glutamate together with DA, respectively (Yamaguchi *et al.*, 2011; Tritsch *et al.*, 2012).

To orchestrate the reward-seeking behavior, the VTA has two principal DA output pathways, one projecting to the NAc (mesolimbic) and the other projecting to the PFC (mesocortical) (**Fig. 20**). The activation of the **mesolimbic** axis triggers the enhancement of extracellular DA in the NAc, producing the reinforcement phenomena. This dopaminergic increase positively correlates with the intensity of “pleasure” subjects experience when taking drugs (Volkow *et al.*, 2010; Oleson and Cheer, 2012). The activation of the **mesocortical** network is essential to the regulation of cognitive control, motivation, and emotional response (Volkow *et al.*, 2017). In addition, the VTA sends glutamatergic and

GABAergic projections to the NAc and to the lateral habenula to modulate both positive and negative reinforcements (Stamatakis *et al.*, 2013; Qi *et al.*, 2016). Finally, VTA dopaminergic neurons also innervate other regions, including the amygdala and hippocampus, where aversive states and contextual associations of drug-related cues are processed, respectively (Koob and Volkow, 2010).

The VTA receives excitatory inputs from several brain regions, including glutamatergic projections from the laterodorsal tegmental nucleus and the lateral habenula (**Fig. 20**). The projections from the **laterodorsal tegmental nucleus** preferentially synapse on DA VTA neurons projecting to NAc, and its activation triggers rewarding effects. In contrast, glutamatergic **lateral habenula** neurons innervate DA VTA neurons that project to the PFC, inducing aversive behaviors (Lüscher, 2016). Moreover, the VTA receives inhibitory inputs of GABAergic projections from the **rostromedial tegmental nucleus** ("tail" of the VTA), exerting an inhibitory control over the DA VTA-NAc pathway (Cooper et al., 2017). In turn, the rostromedial tegmental nucleus activity is enhanced by the excitatory projections from lateral habenula (Pistillo et al., 2015). Other brain regions, such as the PFC, NAc, amygdala, ventral pallidum, and LH, also send projections to the VTA (Lüscher, 2016) conforming, with the explained afferents and efferents, a complex local microcircuit within the mesocorticolimbic network (**Fig. 20**).

3.2.2 Nucleus accumbens

The NAc is primarily characterized by its substantial inputs from the mesolimbic DA system originated in the VTA and limbic structures such

as the amygdala, hippocampus, midline thalamus, and certain regions of the PFC (Basar et al., 2010). The release of DA into this midbrain area trigger goal-directed behaviors to promote reward or avoidance. Therefore, the NAc is proposed to be a key node of the mesolimbic DA circuitry that integrates motivational and reward-related behaviors (Volkow *et al.*, 2017).

The NAc forms the main part of the so-called ventral striatum and is subdivided into two functional subregions known as the core (central part) and the shell (surrounding the core medially, ventrally, and laterally (**Fig. 21**). The **core** has been conceptualized to be involved in the acquisition of reward-cue associations, reactions to motivational stimuli, impulsive choices, and initializing motor actions. However, the **shell** has been proposed to be engaged in reward prediction, affective processing, and drug relapse (Salgado and Kaplitt, 2015; Scofield *et al.*, 2016).

Regarding the cytoarchitecture, the principal neurons of the NAc are GABAergic medium spiny neurons (**MSNs**) that form more than 95% of the total population. The remaining ~5% is composed of GABAergic and cholinergic interneurons that modulate the inhibitory projections. In addition, the MSNs are subdivided into two subtypes based on the receptors that they express: DA type-1 receptor (D1R)-expressing MSNs that also contain dynorphin and D2R-expressing MSNs that also contains enkephalin (Scofield *et al.*, 2016). NAc D1- and D2-MSNs have been considered to have different anatomical connectivity and, therefore, different functions.

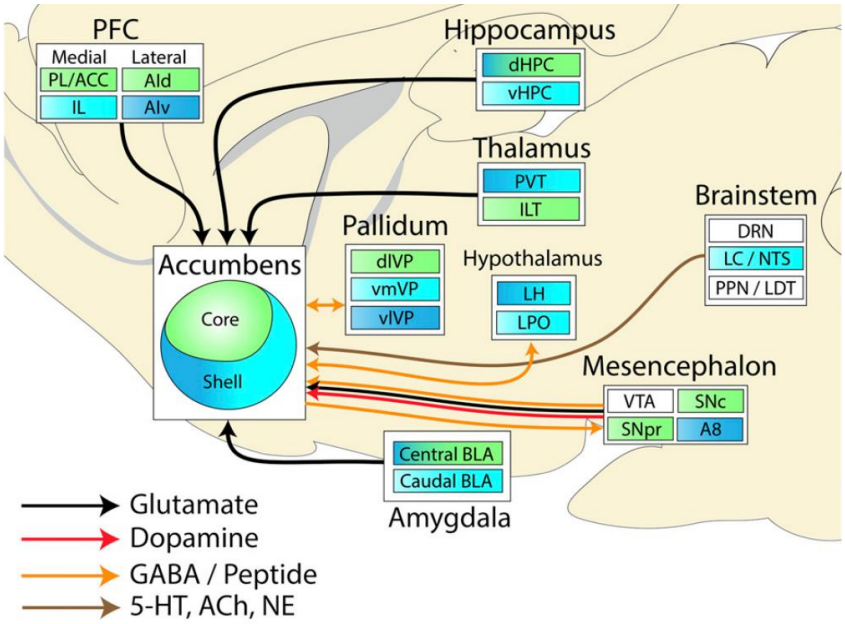

Figure 21. **Schematic diagram of the NAc connectivity.** The NAc receives inputs from cortical, thalamic, midbrain, and brainstem structures. In turn, it sends projections to other basal ganglia nuclei (VP and substantia nigra pars reticulata), nuclei in the mesencephalon, the hypothalamus, and the extended amygdala. These projections have been shaded-coded as projecting to the NAc core (green), medial NAc shell (light blue), or lateral NAc shell (dark blue). Regions that project uniformly throughout the NAc are marked white. A8, retrorubral area; ACC, anterior cingulate cortex; AId, dorsal anterior insular; AIv, ventral anterior insular; dHPC, dorsal hippocampus; dlVP, dorsolateral ventral pallidum; DRN, dorsal raphe nucleus; IL, infralimbic cortex; ILT, interlaminar nuclei of the thalamus; LC, locus coeruleus; LH, lateral hypothalamus; LPO, lateral preoptic area; NTS, nucleus of the solitary tract; PL, prelimbic cortex; PPN, pedunculopontine nucleus; PVT, paraventricular nucleus of the thalamus; vlVP, ventrolateral ventral pallidum; vmVP, ventromedial ventral pallidum; SNc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata. Extracted from (Scofield *et al.*, 2016).

Classically, D1-MSNs of the dorsal striatum are key components of the so-called **direct pathway** and connect with basal ganglia nuclei, the

substantia nigra pars reticulata (SNpr), and the internal globus pallidus (GPi), promoting goal-directed behaviors. D2-MSNs are part of the **indirect pathway** and send outputs to the external globus pallidus (GPe), decreasing goal-directed behaviors (**Fig. 22**) (Bock *et al.*, 2013; Klawonn and Malenka, 2018). However, recent studies with optogenetics and tracing tools have demonstrated that this model is oversimplified. Indeed, a significant proportion of D1-MSNs also projects to the ventral pallidum, whereas some D2-MSNs project directly to the thalamus, comprising the classical indirect and direct pathways, respectively (**Fig. 22**) (Kupchik and Kalivas, 2017; Klawonn and Malenka, 2018).

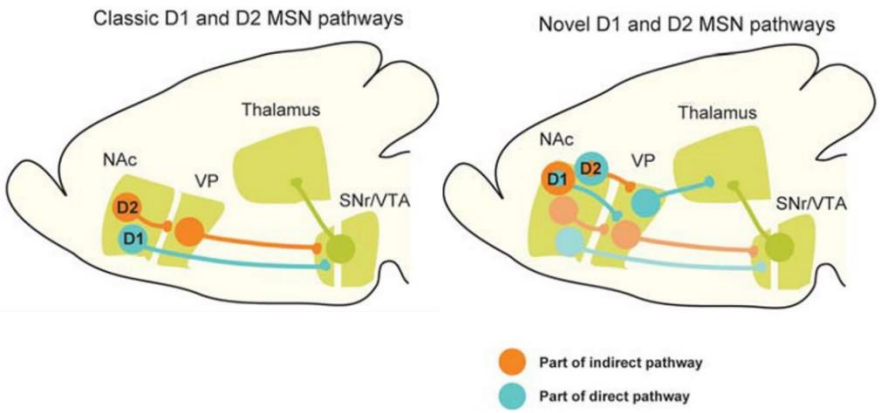

Figure 22. Schematic diagram of classic and novel views of NAc D1- and D2-MSNs anatomical connectivity. Nucleus accumbens (NAc) D1 medium spiny neurons (MSNs) are commonly thought to project directly to midbrain structures, primarily the ventral tegmental area (VTA), whereas NAc D2 MSNs are thought to project primarily to the ventral pallidum (VP). Current research suggested a re-wiring of the classic connections of NAc D1- and D2-MSN. SNr, substantia nigra pars reticulata. Adapted from (Klawonn and Malenka, 2018).

Before reaching other areas, outputs from the NAc first reach the ventral pallidum. The NAc core projects primarily to the dorsolateral portion of the ventral pallidum, which projects to the subthalamic nucleus and SNpr. In contrast, the NAc shell mainly innervates the ventromedial ventral pallidum, which further innervates VTA, hypothalamus, and PFC areas (Salgado and Kaplitt, 2015) (**Fig. 23**).

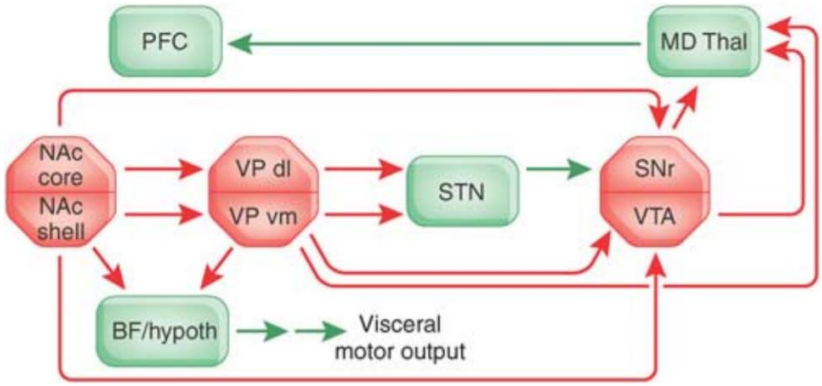

Figure 23. **Major projections from nucleus accumbens-ventral pallidum circuits based on NAc topography.** Red indicates inhibitory structures and pathways, whereas green indicates excitatory connections. MD Thal, mediodorsal thalamic nucleus; NAc, nucleus accumbens; PFC, prefrontal cortex; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VP dl/vm, ventral pallidum, dorsolateral, and ventromedial; VTA, ventral tegmental area. Extracted from (Sesack and Grace, 2010).

Furthermore, there is reciprocal connectivity between NAc and VTA that provides additional complexity to the mechanisms underlying reward-related behaviors. Optogenetic stimulation has demonstrated that NAc shell neurons synapse onto VTA DA neurons suppressing behavioral output (**direct loop**), as well as onto VTA GABAergic neurons leading to disinhibition of DA neurons projecting back to the NAc

(**indirect loop**) (**Fig. 24**) (H. Yang *et al.*, 2018). Hyperactivation of this NAc-VTA pathway increases the firing of the VTA DA neurons and could mediate the exacerbated DA activity produced by drugs of abuse (Klawonn and Malenka, 2018).

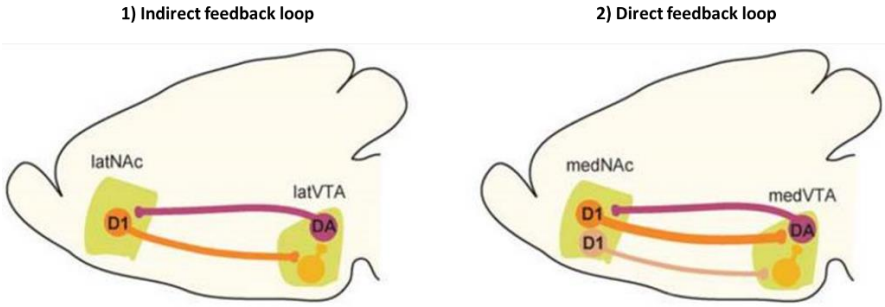

Figure 24. **Simplified diagram of the feedback connectivity between NAc and VTA. 1) Indirect feedback loop:** lateral shell nucleus accumbens (NAc) D1 MSNs synapse on ventral tegmental area (VTA) GABAergic neurons, which exert inhibitory influence over NAc lateral-projecting DA neurons. These neurons project back to the lateral NAc promoting reward-related behaviors. **2) Direct feedback loop:** medial shell NAc D1 MSNs synapse on medial VTA DA neurons that project back to the medial NAc suppressing behavioral output. Adapted from (Klawonn and Malenka, 2019).

Finally, the NAc MSNs receive inputs from the mesolimbic pathway emerging from the VTA and other cortical and subcortical structures, such as the PFC, amygdala, hippocampus, and thalamus (Sesack and Grace, 2010) transferring different types of information. Integrating all this information suggests that NAc is a critical node of the mesolimbic DA circuitry that integrates motivational and reward-related behaviors. However, the functioning of NAc circuitry is complex and heterogeneous, and it can be altered by experiences such as addiction or depression (Salgado and Kaplitt, 2015; Serafini *et al.*, 2020).

3.2.3 Prefrontal cortex

The PFC is a brain area involved in several physiological functions related to the inhibitory control of behavior. Impairment of this executive brain area leads to a loss of self-control, driving compulsive behaviors, such as drug abuse and food overconsumption (Goldstein and Volkow, 2011; Volkow and Baler, 2014). The PFC includes the mPFC and the OFC (Maldonado *et al.*, 2021b).

The **mPFC** cytoarchitecture consists of 80% excitatory glutamatergic pyramidal projection neurons and 20% GABAergic inhibitory interneurons (Pistillo *et al.*, 2015). In addition, the PFC has a **laminar organization** typical of cortical areas. The excitatory pyramidal neurons located in superficial layers (L2/3) create intracortical circuits with other pyramidal and GABAergic neurons. In contrast, the pyramidal neurons located in deep layers (L5/6) send excitatory projections to subcortical areas, including VTA and NAc. In turn, deep pyramidal neurons in L5 and 6 receive afferent projections from VTA dopaminergic neurons, while pyramidal neurons in L2, 3, and 5 receive inputs from other cortical areas, thalamus, basolateral amygdala (BLA), and hippocampus. Thus, the VTA-PFC relationship works as a feedback loop that evaluates the salience and motivational significance of drug- and food-associated contexts and stimuli (Douglas and Martin, 2004).

It is worth highlighting that the mPFC is relatively comparable between humans and rodents regarding similarities in connections and functions (Jasinska et al., 2015). It has been demonstrated that the human dorsolateral PFC is equivalent to the rodent prelimbic cortex (PL) and the ventral component of the PFC corresponds to the rodent

infralimbic cortex (IL) (Koob and Volkow, 2016) (**Fig. 25**). These mPFC subregions are reciprocally interconnected but have dissociable connectivity with other areas of the reward system. Indeed, the **PL** (dorsal mPFC) projects predominantly to the NAc core and basolateral amygdala, whereas the **IL** (ventral mPFC) innervates almost exclusively the NAc shell and several amygdala nuclei different from the basolateral area (Jasinska *et al.*, 2015; Moorman *et al.*, 2015). Both PL and IL cortices could drive or inhibit drug-seeking depending on the behavioral context, the type of drug, and the previous history of drug consumption, implying that several subcircuits within each of these mPFC areas may have distinct behavioral functions (Maldonado *et al.*, 2021b). Particularly, PL activity reflects a learning association between environmental cues and conditioned fear-related behaviors, such as aversive foot-shock, associating the punishment with the reward (Jasinska *et al.*, 2015).

Other sub-regions within the mPFC also play functional roles in the transition to addiction, such as the ACC and the insular cortex. The **ACC** has been mainly involved in inhibitory control/emotion regulation (Volkow *et al.*, 2019). Moreover, the ACC is part of the anterior default mode network engaged in the interoceptive processes of addiction. The **insular cortex** corresponds to the agranular insula in rodents (**Fig. 25**) and has also been shown to be involved in the maintenance of drug addiction (Maldonado *et al.*, 2021b). Bidirectional communication between the ACC and the insula suggests a role in integrating autonomic and visceral information with emotive and motivational information that enables the conscious awareness of internal urges

(Volkow *et al.*, 2019). Accordingly, the anterior insular cortex is activated during drug craving, and lesions in this area help to prevent drug relapse in both humans (Naqvi *et al.*, 2007) and rodents (Rotge *et al.*, 2017).

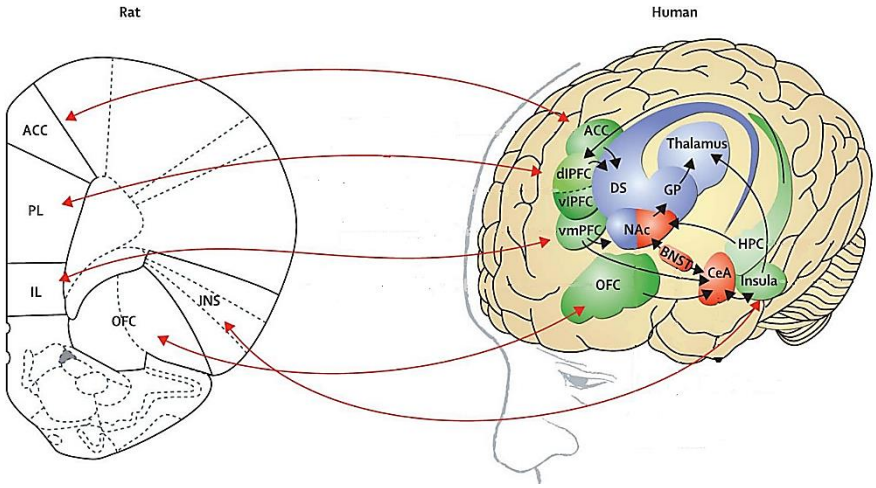

Figure 25. Correspondence between rat and human cortical regions relevant to the transition to addiction. Color-shaded areas correspond to three functional domains: reward and incentive salience (basal ganglia and thalamus) [blue], negative emotional states and stress (extended amygdala) [red], and craving, impulsivity, and executive function (PFC, insula, and allocortex) [green]. ACC: anterior cingulate cortex, PL: prelimbic cortex, IL: infralimbic cortex, OFC: orbitofrontal cortex, INS: insula, dlPFC: dorsolateral prefrontal cortex, vlPFC: ventrolateral prefrontal cortex, vmPFC: ventromedial prefrontal cortex, DS: dorsal striatum, GP: globus pallidus, NAc: nucleus accumbens, BNST: bed nucleus of the stria terminalis, CeA: central nucleus of the amygdala, HPC: hippocampus. Adapted from (Koob and Volkow, 2016).

Finally, the **OFC** is also relatively equivalent in humans and rodents (**Fig. 25**). It has been recently reported that this cortical area has a relevant role in associative learning, cue-reactivity, and goal-directed decision-

making (Kalon *et al.*, 2016; Lüscher *et al.*, 2020). Furthermore, the OFC, together with the insular cortex, is also involved in the processing of palatability. Primary neurons allocated in the insular cortex process taste and smell inputs and project these signals to secondary neurons found in the OFC, which integrates this sensory information (Rolls, 2012). However, the possible differential role of the sub-regions of this cortical area within the olfactory system has not yet been clarified (Maldonado *et al.*, 2021b).

3.3 Food addiction

3.3.1 Definition

Theron Randolph first described the term food addiction in 1956, who suggested that certain foods produce a "common pattern of symptoms descriptively similar to those of other addictive processes" (Randolph, 1956). Since then, research has focused upon elucidating the addictive biological and behavioral similarities between the effects of drugs and food. Indeed, the concept of food addiction has emerged as a topic of interest since the number of animal and human studies investigating the neurobiological mechanisms involved in this pathology has dramatically augmented in the last 10 years (Meule, 2015; Fernandez-Aranda *et al.*, 2018) (**Fig**. **26**).

Today's society consumes palatable foods rich in sugar, carbohydrates, fat, or even salt not only to satiate hunger but also for pleasure (Alonso-Alonso *et al.*, 2015). Consequently, food addiction embraces the idea that specific foods (highly processed, highly palatable, and highly caloric) have an addictive potential contributing to overeating.

However, within the scientific community, there are controversies concerning the classification of this disorder as a "chemical" (substance-based) or a "behavioral" (non-substance-based) addiction (Meule, 2019).

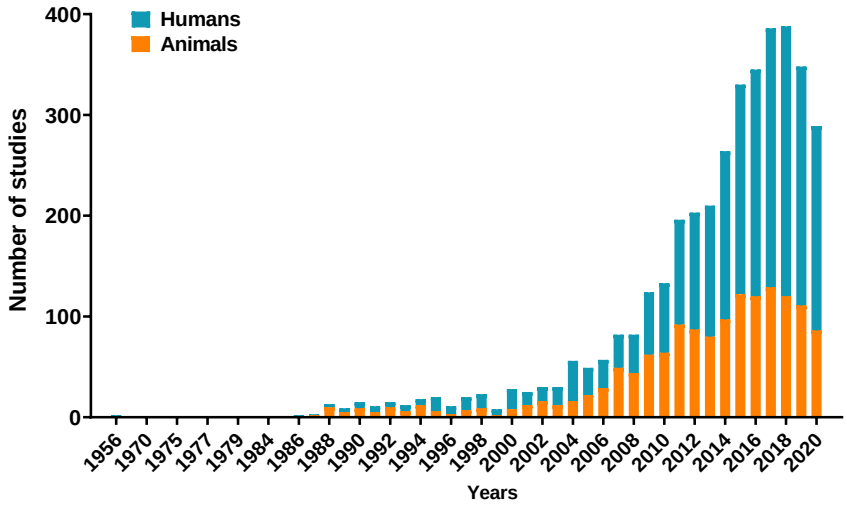

Figure 26. **Number of animal and human studies published on food addiction from 1947 to 2020.** Extracted from PubMed May 2021.

Regarding a biochemical approach to addiction, several studies showed that highly palatable food containing high-fat and/or sugar is capable of promoting addiction-like behaviors and neuronal changes that parallel drug addiction (Ifland *et al.*, 2015; Schulte and Gearhardt, 2017). Accordingly, the use of the term **"food addiction"** appears appropriate as a substance-based addiction, like nicotine, cocaine, or alcohol, among others (Serafine Katherine *et al.*, 2021). Moreover, the reliance on highly palatable foods to cope with stress and negative emotions may be considered components of food addiction similar to those observed in drug abusers to manage anxiety and depression

(Parylak *et al.*, 2011; Carter *et al.*, 2019). Indeed, the past lockdown months have impacted many people's mental health and led to detrimental behaviors, including substance and food addictions, to manage the stress and uncertainty brought by the COVID-19 pandemic (Avena *et al.*, 2021). In contrast, other models describe food overconsumption as a behavioral addiction to the act of eating itself, like those addictions that are non-substance related, such as gambling. In this context, the term **"eating addiction"** may be more appropriate (Hebebrand *et al.*, 2014). This statement is based on the unconfirmed presence of specific chemical substances with addictive properties in foods that can lead to the development of a substance use disorder.

Despite these controversies concerning the concept of food addiction, it seems widely accepted that embracing an addiction perspective on food has practical implications for the prevention and treatment of eating disorders and obesity (Meule, 2019). Altogether, the question of whether food addiction is a valid concept is still under debate, and apparently, this question will not be resolved anytime soon. Therefore, more scientific research in this field is required to clarify these controversies and provide adequate prevention and treatment for food addiction.

3.3.2 Diagnostic tool: Yale Food Addiction Scale

Food addiction has not been recognized as a disorder in the most recent version of the Diagnostic and Statistical Manual of Mental Disorders, the DSM-5. Thus, the Yale Food Addiction Scale (YFAS) was developed as a diagnostic tool to collect the symptomatology and assess the severity of food addiction (Gearhardt *et al.*, 2009).

The last version of the YFAS, **YFAS 2.0**, is a 35-item report that provides a method of diagnosing food addiction based on the criteria included on the last version of the DSM for substance dependence (Gearhardt *et al.*, 2016). The DSM-5 measures severity on a continuous scale from mild (2-3 symptoms endorsed), moderate (4-5 symptoms endorsed), and severe (6 or more symptoms endorsed) out of 11 total symptoms (**Table 7**) (Gordon *et al.*, 2018). YFAS 2.0 also uses mild, moderate, or severe categories for the diagnostic threshold (Gearhardt *et al.*, 2016). Moreover, the use of the YFAS 2.0 in obese individuals appears to be an appropriate method to diagnose food addiction before and after surgical interventions (Koball *et al.*, 2021). In patients seeking **bariatric surgery**, food addiction can impede weight loss in preoperative dietary interventions and may be related to mood and anxiety disorders or addictive behaviors (Benzerouk *et al.*, 2018; Clark *et al.*, 2019). Thus, it seems accurate to measure the construct of food addiction in these patients preoperatively to optimize surgical outcomes.

In summary, the YFAS is currently the best reliable and validated tool for evaluating food addiction based upon modified DSM-5 criteria for substance use disorders (Schulte and Gearhardt, 2017), serving as a clinically useful measure of food addiction severity in patients pursuing bariatric surgery (Koball *et al.*, 2021).

DSM-5 SUD criteria	mYFAS 2.0 question
Substance taken in larger amount and for longer period than intended	I ate to the point where I felt physically ill.
Persistent desire or repeated unsuccessful attempts to quit	I tried and failed to cut down on or stop eating certain foods.
Much time/activity to obtain, use, recover	I spent more time feeling sluggish or tired from overeating.
Important social, occupational or recreational activities given up or reduced	I avoided work, school or social activities because I was afraid I would overeat there.
Use continues despite knowledge of adverse consequences	I kept eating in the same way even though my eating caused emotional problems.
Tolerance	Eating the same amount of food did not give me as much enjoyment as it used to.
Characteristic withdrawal symptoms; substance taken to relieve withdrawal	If I had emotional problems because I had not eaten certain foods, I would eat those foods to feel better.
Continued use despite social or interpersonal problems	My friends or family were worried about how much I overate.
Failure to fulfil major role obligations	My overeating got in the way of me taking care of my family or doing household chores.
Use in physically hazardous situations	I was so distracted by eating that I could have been hurt (e.g. when driving a car, crossing the street and operating machinery).
Craving, or a strong desire or urge to use	I had such strong urges to eat certain foods that I could not think of anything else.
Use causes clinically significant impairment	I had significant problems in my life because of food and eating. These may have been problems with my daily routine, work, school, friends, family or health.
Use causes clinically significant distress	My eating behaviour caused me much distress.

Table 7. The Substance Use Disorder criteria adapted from the DSM-5 and the equivalent modified Yale Food Addiction Scale 2.0 (mYFAS 2.0) questions. DSM-5: Diagnostic and Statistical Manual of Mental Disorders-5, SUD: substance-use disorder. Adapted from (Schulte and Gearhardt, 2017).

3.3.3 Food addiction prevalence and comorbid diseases

According to the YFAS, the prevalence of food addiction in the adult population is 19.9% (Pursey *et al.*, 2014). The percentage of healthy BMI individuals affected is between 2-12% (Fernandez-Aranda *et al.*, 2018), but this percentage raises in subjects with obesity (18-24%) and eating disorders, such as binge eating or bulimia nervosa (50% and 85%, respectively) (Davis *et al.*, 2011; Hilker *et al.*, 2016; Burrows *et al.*, 2017). The ranging prevalence of food addiction seems to be explained by the existing overlap between assessment items in YFAS and criteria for other eating disorders, especially those addressing the feeling of loss of control and excessive eating (Oliveira *et al.*, 2021). Interestingly, food addiction seems more prevalent in females than males despite that drug addiction seems to principally affect men (Pursey *et al.*, 2014).

To date, several studies have found substantial comorbidity between food addiction and other **eating disorders**, such as binge eating disorder (BED) and bulimia nervosa (Oliveira *et al.*, 2021). Indeed, 56.8% of binge eaters met YFAS criteria for food addiction (Gearhardt *et al.*, 2013). Nevertheless, behavioral and theoretical features differentiate both disorders, and not all the individuals who met food addiction criteria (30%) are also BED patients, confirming that they are two independent clinical entities (Davis *et al.*, 2011). Furthermore, food addiction has also been linked with overweight and **obesity**. Indeed, symptoms of food addiction are more prevalent among overweight and obese adults (24.9%) compared to those with normal BMI (11.1%) (Pursey *et al.*, 2014). Importantly, increasing studies addressing food

addiction focused on severe obesity in patients seeking bariatric surgery (Koball *et al.*, 2021). The high prevalence of food addiction among individuals with morbid obesity seeking bariatric surgery varies from 30 to 50% (Oliveira *et al.*, 2021). Thus, the study of food addiction is of particular interest for understanding the causes and management of obesity. In addition, patients with BED, bulimia nervosa and obesity share a significant number of comorbidities as those reported by individuals with severe levels of FA, such as emotional eating, loss of control, social distress, and cognitive impairment (Rosenbaum and White, 2015; Ivezaj *et al.*, 2016; Oliveira *et al.*, 2021), suggesting that emotional stability is one main driver for compulsive eating behaviors.

3.4 Dynamics in the transition to addiction: stages of the food addiction cycle

Addiction is a recurring cycle composed of three stages that repeat over and over until there is a shift from controlled consumption to the loss of control. In the case of food addiction, this cycle starts with an acute intoxication or binge eating episode, followed by the emergence of an adverse effect that leads to the failure of inhibitory control (**Fig. 27**) (Koob and Volkow, 2010, 2016). These three stages of addiction have their neurobiological correlates in neurocircuits involved in reward learning, emotional processing, and inhibitory control, respectively: the basal ganglia, the extended amygdala, and the PFC (Moore *et al.*, 2017b, 2018). The transition from controlled to compulsive food intake involves neuroplasticity changes in these brain structures that may begin with sensitization of the mesolimbic DA system (Piazza and Deroche-Gamonet, 2013).

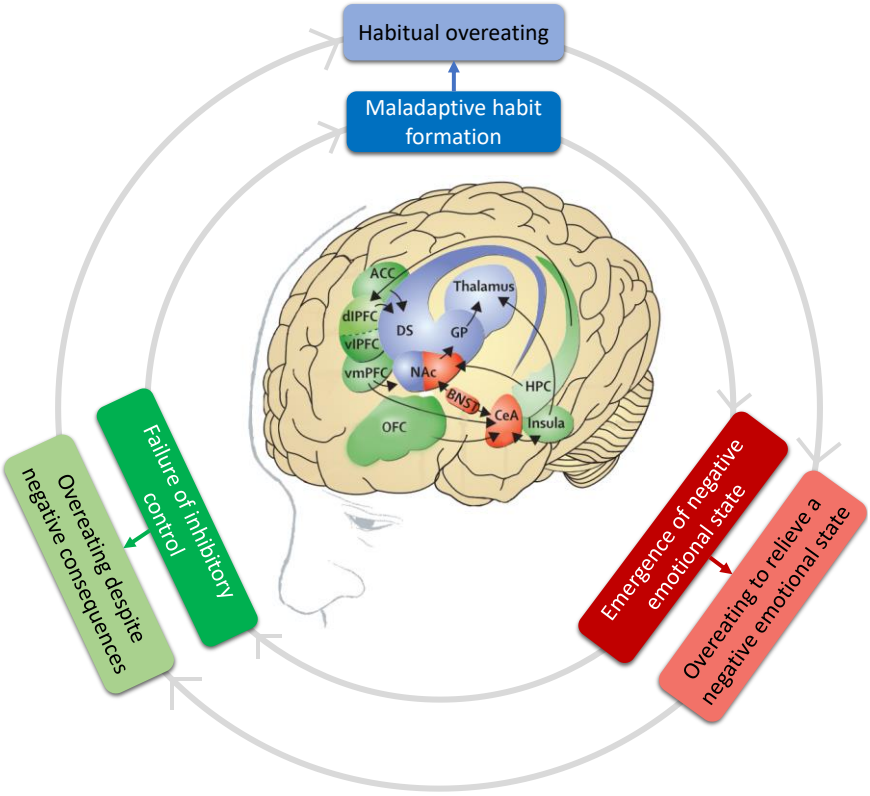

Figure 27. The addiction cycle is conceptualized in three stages with the corresponding brain areas involved. The neurocircuitry model has three different functional domains, in which each dysfunction contributes to compulsive eating behavior: habitual overeating (reward and incentive salience: basal ganglia [blue]), overeating to relieve a negative emotional state (negative emotional states and stress: extended amygdala [red]), and overeating despite negative consequences (craving, impulsivity, and executive function: PFC, insula, and allocortex [green]). Adapted from (Koob and Volkow, 2016; Moore *et al.*, 2018).

3.4.1 Habitual overeating: maladaptive habit formation

Initially, the individual performs a sporadic acute intoxication of food. This binge-eating episode activates the same brain substrates that mediate the positive reinforcing effects of natural rewards, like the

increased DA release in the NAc shell (Di Chiara and Imperato, 1988). After a prolonged habitual overeating history, a maladaptive habit emerges with the association of environmental stimuli (cue) with food availability in the so-called **conditioned reinforcement** phenomenon. If the environmental cue appears repeatedly paired with the presence of food, the cue itself becomes eye-catching, and this phenomenon is termed **incentive salient** (Koob and Volkow, 2016). Both conditioned reinforcement and incentive salience can enormously increase the desire to eat and maintain food-seeking even when food is not present or lacks nutrients in the body, building the habit (Velázquez-Sánchez *et al.*, 2015; Moore *et al.*, 2017b). Furthermore, compulsive seeking behavior is observed in mice trained in operant conditioning maintained by palatable food, **unconditioned stimulus**, associated with a cue light, **neutral stimulus**. When the unconditioned stimulus is paired repeatedly with the neutral stimulus, the food-associated stimulus is conditioned, evoking a food-seeking response even if the original unconditioned stimulus is not available anymore (Mancino *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020).

This maladaptive habit formation results from a learning process where voluntary actions become habit-based behaviors through striatal-dependent mechanisms (Stahl, 2013). The modulatory role of the glutamate inputs coming from the mPFC into the NAc weakens strength due to the neuroplasticity processes associated with the repetition of the behavior. In contrast, glutamate projecting from the OFC to the dorsal striatum strengthen, promoting the shift to habit formation (Lüscher *et al.*, 2020). In accordance, preclinical evidence

reported an imbalance in the activity of these cortico-striatal circuits, with more activity in OFC-dorsal striatum connections and less engagement of the mPFC-ventrolateral striatum circuitry in rats showing compulsive methamphetamine taking (Hu *et al.*, 2019). Moreover, recent neuroimaging studies have shown a reduced activation of the caudate nucleus of the striatum (involved in goal-directed actions) and an enhanced activity of the putamen (involved in habit responding) to palatable food (Babbs *et al.*, 2013; Moore *et al.*, 2017b). Thus, the OFC has a relevant role in associative learning and goal-directed decision making (Kalon *et al.*, 2016; Lüscher *et al.*, 2020), promoting food-stimuli reactivity to maintain compulsive seeking behavior associated with food craving.

3.4.2 Overeating to relieve a negative emotional state: the emergence of a negative affect

As individuals progress towards overeating behaviors, palatable food progressively produces long-term neuroadaptations in the brain reward system and shifts from being strongly wanted to be strongly needed. Consumption of palatable food loses the rewarding hedonic properties in favor of promoting food intake to prevent or ameliorate negative states (anxiety, depression, or irritability) when those foods are no longer available (Parylak *et al.*, 2011). In agreement, dietary restraint in humans after overeating resulted in negative emotions such as irritability, nervousness, and intense anxiety (Greeno and Wing, 1994). This negative reinforcement promotes, even more, the overeating behavior and is considered the "**dark side**" of the addiction cycle (Koob, 2013, 2015).

The negative reinforcement mechanism is hypothesized to derive from the dysregulation of neurotransmitters within the **extended amygdala** (Moore *et al.*, 2017b). This stage includes diminished reward neurotransmission, like a decreased DA level and activation of brain **stress systems** in the extended amygdala that regulates the induction of anxiety and depressive-like behaviors (Maldonado *et al.*, 2020). Both the hypothalamic-pituitary-adrenal axis and the brain stress system are dysregulated in this stage, promoting the release of corticotropin-releasing factor (CRF) (Koob, 2017). Indeed, rats exhibited increased anxiety and depressive-like behaviors during withdrawal of palatable food accompanied by increased expression of CRF in the central amygdala (Cottone *et al.*, 2009). In agreement, morphometric changes in subcortical brain regions, including the amygdala, have been found in humans with alcohol use disorder (AUD). Indeed, the amygdala volume showed a positive association with anxiety and negative urgency in AUD patients that corroborates the amygdala's involvement in the dark side of addiction (Tomasi *et al.*, 2021).

3.4.3 Overeating despite aversive consequences: failure of inhibitory control

The repeated consumption of palatable food leads to a loss of inhibitory control over the food intake behavior, mainly caused by dysfunctions in multiple frontostriatal circuitries. The loss of control over food intake results in **compulsive behavior**, consisting of food consumption despite negative consequences that typically suppress food intake (Deroche-Gamonet *et al.*, 2004). The negative consequences associated with compulsive food intake, such as

physical, psychological, and social problems, are the main cause of relapse into unhealthy eating habits (Moore *et al.*, 2018).

The PFC mainly regulates the frontostriatal circuitries implied in the loss of inhibitory control. Two complementary frontostriatal circuits have been identified depending on whether their recruitment promotes or difficult the transition to addiction-like behaviors: the Go and the Stop circuits (**Fig. 28**). The **Go circuit** is mediated by OFC neurons projecting to the medial striatum and strengthening activity when the loss of control is evidenced (Pascoli *et al.*, 2018; Hu *et al.*, 2019). Otherwise, the **Stop circuit** is mediated by the neuronal projection from the dorsolateral PFC to the NAc, resulting in weakening neuronal activity when the inhibitory control is lost, and compulsive consumption appears. A potentiation of glutamatergic input to this Stop circuit produced resilience toward compulsive drug-seeking in different murine models (Bock *et al.*, 2013; Chen *et al.*, 2013; Hu *et al.*, 2019). Thus, the unbalanced of the go and stop circuitries is more pronounced through the progression of the addictive cycle. On one side, there is a hypoactivation of the prefrontal circuits that leads to the disinhibition of reward and stress systems, and on the other hand, a hyperactivation of striatal areas is more sensitive to food-related cues (Koob and Volkow, 2016).

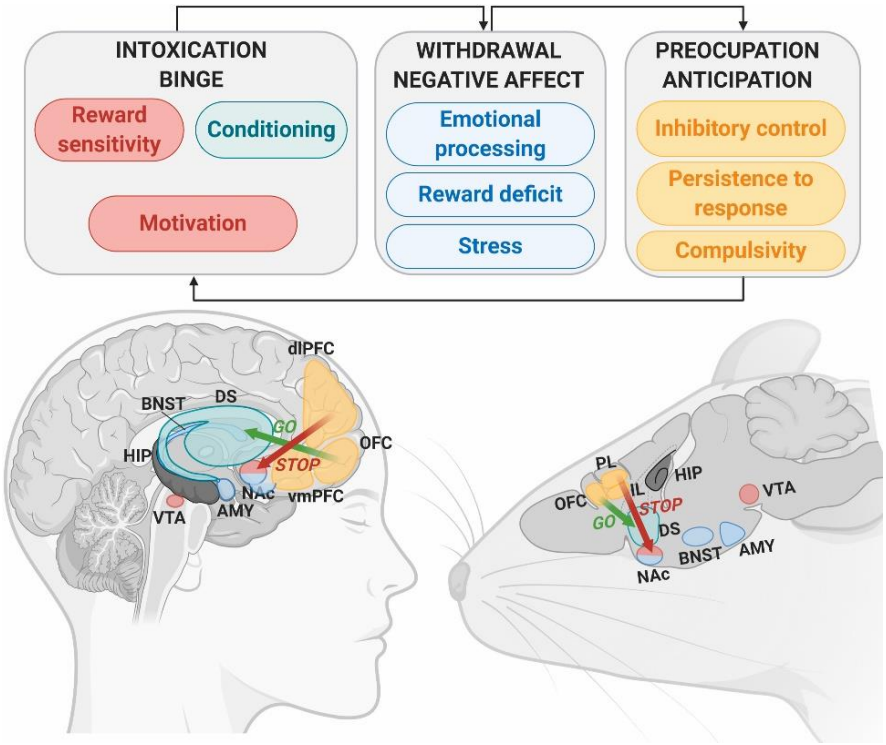

Figure 28. Go and Stop circuits: two complementary frontostriatal pathways involved in the loss of inhibitory control in addiction. Compulsive behavior is related to the connectivity strength of the medial PFC glutamatergic projections to the NAc. (Maldonado *et al.*, 2021b).

The loss of inhibitory control over food consumption has also been proved in laboratory animal models. After operant training to obtain palatable food, animals classified as addicted showed a compulsive eating behavior even in aversive conditions, such as the presence of electrical foot-shocks or during extinction protocols when only reward-associated cues are presented (Deroche-Gamonet *et al.*, 2004; Everitt *et al.*, 2008; Mancino *et al.*, 2015; Velázquez-Sánchez *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020; Maldonado *et al.*, 2021b). Notably, one of these studies showed that the inhibition of mPFC projections to

the NAc core (Stop circuit) resulted in the compulsive consumption of palatable food and the enhancement of addictive-like behaviors (Domingo-Rodriguez *et al.*, 2020).

3.5 The endocannabinoid system in eating behavior

The endocannabinoid system is a critical player in regulating energy balance and eating behavior in peripheral tissues and the central nervous system (Di Marzo, 2008; Heyman *et al.*, 2012). Remarkably, the ECS exerts widespread modulatory influence acting on both homeostatic and hedonic control systems of food intake (Parsons and Hurd, 2015; Spanagel, 2020) (previously described in sections 1.3.4 and 1.3.5). Thus, the extended role of this endogenous system in the control of food intake supports the hypothesis that alterations in the ECS may lead to the development of eating disorders, like food addiction (C. D'Addario *et al.*, 2014).

3.5.1 The endocannabinoid system in food addiction

Evidence of alterations in the ECS has been found in patients with obesity and eating disorders, such as BED or anorexia nervosa (Di Marzo and Matias, 2005; Gérard *et al.*, 2011). Indeed, human genetic studies have reported a positive association between eating disorders and specific polymorphisms of genes encoding for different components of the ECS system, such as CB1R (Monteleone *et al.*, 2009) and CB2R (Ishiguro *et al.*, 2010; Onaivi *et al.*, 2012). However, the mechanisms about the modulatory role of the ECS in food addiction are poorly known.

Previously in our laboratory, the validation of a food addiction mouse model performed allows us to study the involvement of the ECS in food addictive behavior (Mancino *et al.*, 2015). We observed that long-term operant training to obtain highly palatable food produces epigenetic changes in the expression of the **CB1R** gene, with the subsequent increase in CB1R protein in mice classified as food addicted. Afterward, both pharmacological (rimonabant) and genetic manipulation tools (deletion of CB1R) reduced the percentage of animals that reached the addiction criteria. In accordance, clinical trials with the CB1R antagonist rimonabant have already demonstrated efficacy at reducing food intake in food-addicted patients (Hagmann, 2008) and rodents (Maccioni *et al.*, 2008; Blasio *et al.*, 2014). However, rimonabant produces important side effects, such as depression and suicidal ideation, that make it inappropriate as a therapeutic approach (Galaj and Xi, 2019).

A recent study in our group has corroborated the involvement of **CB1R** in food overconsumption and, more importantly, has elucidated the specific cell type in which CB1R is exerting this addictive-like behavior effect. We reported that the lack of CB1R in dorsal telencephalic glutamatergic neurons prevents the development of food addiction-like behavior. CB1R modulates the glutamatergic projections from the mPFC to the NAc core, ultimately enhancing extracellular DA levels in the NAc and affecting brain reward processes, facilitating the avoidance behavior (NO GO response) and promoting a resilient phenotype to food addiction (**Fig. 29**). In contrast, the chemogenetic

inhibition of the corticolimbic pathway mPFC-NAc induces compulsive food-seeking (Domingo-Rodriguez *et al.*, 2020).

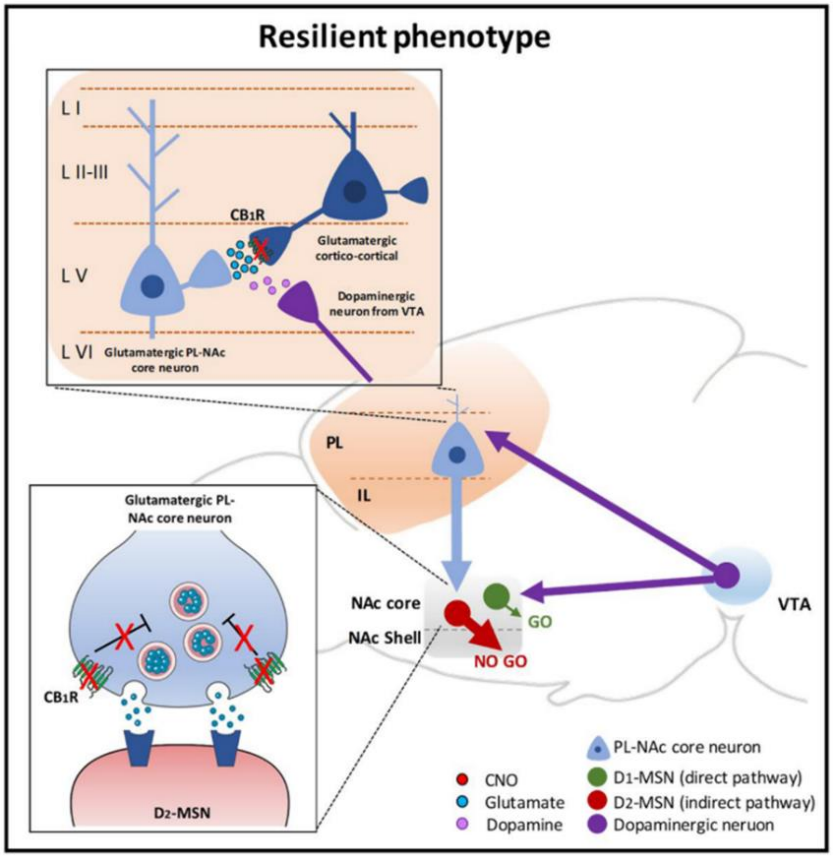

Figure 29. Schematic illustration of the mPFC-NAc core pathway regulation of resilience to develop food addiction. Deletion of the CB1R in dorsal telencephalic glutamatergic neurons increased glutamate release at cortical level increasing excitatory glutamatergic projections from prelimbic neurons to the NAc. This increased cortical glutamatergic transmission stimulated D2-MSN indirect pathway in the NAc core facilitating the avoidance behavior (NO GO response). PL: prelimbic, IL: infralimbic, NAc: nucleus accumbens, VTA: ventral tegmental area, D1-MSN: dopamine D1 medium spiny neuron, D2-MSN dopamine D2 medium spiny neuron, CB1R: cannabinoid receptor type-1. Extracted from (Domingo-Rodriguez *et al.*, 2020).

CB2R was initially regarded as a peripheral cannabinoid receptor. However, recent technological advances in gene detection, alongside the availability of transgenic mouse lines, indicate that CB2Rs are expressed in both neurons and glial cells in the brain, especially under pathological conditions, and are involved in multiple functions at cellular and behavioral levels (Spanagel, 2020). The activation of CB2R in astrocytes or microglia in the NAc attenuates rewarding effects by decreasing DA levels through the modulation of inflammatory cytokine release from these cells (Liu *et al.*, 2017; Manzanares *et al.*, 2018). Moreover, the activation of CB2R in VTA dopaminergic neurons also decreases DA release in the NAc, contributing to the aversive effects of cannabinoids (Jordan and Xi, 2019; Li *et al.*, 2021). Therefore, the balance between CB1R and CB2R activation contributes to the subjective effects of cannabinoids (**Fig. 30**).

CB2R have also been related to the rewarding effects of other drugs of abuse, such as cocaine (Manzanares *et al.*, 2018). Indeed, the enhancement of DA levels induced by cocaine in the NAc was modulated by CB2R. CB2R pharmacological activation reduced cocaine-enhanced NAc DA levels, whereas the blockade of CB2R elevated basal extracellular DA levels in this brain area (Xi *et al.*, 2011). In contrast, mice overexpressing CB2R did not show any differential alteration in NAc DA levels compared with wild-type mice under basal conditions or after cocaine challenge (Aracil-Fernández *et al.*, 2012). Pharmacological manipulations are not always equivalent to genetic alterations. Mice overexpressing CB2R showed reduced cocaine self-administration, cocaine-induced place preference, and locomotor

sensitization (Aracil-Fernández *et al.*, 2012), whereas mutant mice with a deletion of CB2R in dopaminergic neurons showed increased cocaine-induced conditioned place preference (Liu et al., 2017).

Regarding the side effects of CB1R stimulation, further research should be focused on targeting the CB2R for the treatment of food addiction since its low presence in neuronal cells (Shang and Tang, 2017) could avoid the risk of centrally-mediated side effects (Racz *et al.*, 2008a, 2008b).

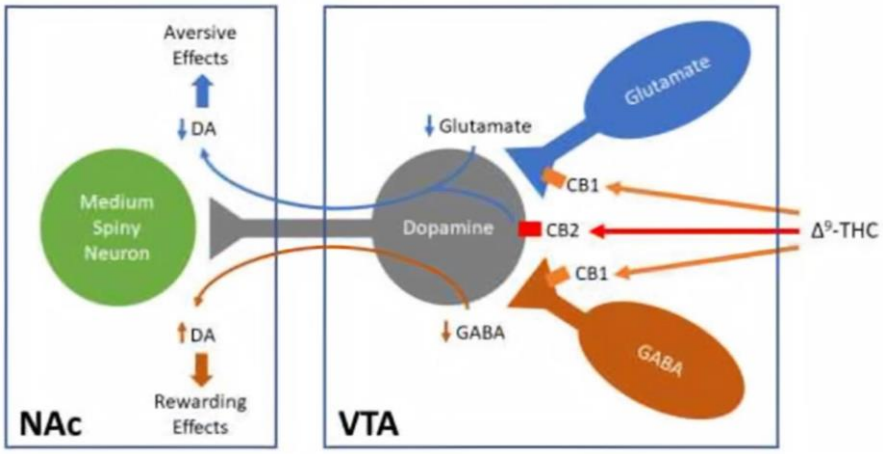

Figure 30. Diagram showing the subjective effects of THC according to CB1R or CB2R activation. THC may produce rewarding effects by binding to CB1R on GABAergic interneurons in the ventral tegmental area (VTA), consequently reducing GABA-mediated inhibition of dopamine (DA) release from the VTA and thereby increasing DA release in the nucleus accumbens (NAc). Conversely, THC may produce aversive effects by activating CB1R on glutamatergic neurons in the VTA or CB2R on DA neurons, thereby inhibiting VTA DA release to the NAc. Extracted from (Jordan and Xi, 2019).

4 Fragile X syndrome

4.1 Fmr1KO mouse for the study of nociceptive processing

Preclinical and clinical evidence suggests that the potential use of the Fmr1KO mouse model of fragile X syndrome (FXS) can be a useful model to investigate. FXS is the leading inherited form of intellectual disability and autism spectrum disorder caused by a CGG trinucleotide expansion in the promoter region of the fragile X mental retardation gene (*FMR1*) that encodes for the fragile X mental retardation protein (FMRP) (Penagarikano *et al.*, 2007). Although the FRMP research is primarily focused on the role of this protein in intellectual disability and neurodevelopmental disorders (Fernández *et al.*, 2013; Hagerman *et al.*, 2017), FMRP impairments include pathological alterations in the somatosensory pathway. Indeed, a prominent behavioral feature of the absence of the FMRP is self-injurious behavior (Symons *et al.*, 2010; Tranfaglia, 2011). Evidence showed that an elevated pain threshold or alterations in pain pathways could underlie the persistence of self-injurious behavior in neurodevelopmental disability patients (Peebles and Price, 2012). These decreased nociceptive responses seem related to altered synaptic transmission along the somatosensory pathway. Accordingly, mice lacking for the *Fmr1* gene (*Fmr1*KO mice) showed deficits in nociceptive sensitization in different models of chronic pain, including inflammatory (Busquets-Garcia *et al.*, 2013) and neuropathic pain responses (Price *et al.*, 2007).

FMRP is also present in sensory nociceptors and brain regions implicated in pain processing (Price *et al.*, 2007) and it is known to contribute to this nociceptive sensitization through the regulation of

mGluR-dependent plasticity (Banerjee *et al.*, 2018). Indeed, under pathological conditions such as neuropathic pain, mGluRs, and especially mGluR5 elicit adaptive changes that contribute to nociceptive plasticity leading to aberrant pain sensitization (Vincent *et al.*, 2016; Xie *et al.*, 2017). Therefore, the loss of FMRP in the Fmr1KO mouse model is a tool for gaining better insight into the development of neuropathic pain.

4.2. General features of fragile X syndrome

FXS is an X-linked dominant disorder caused by a CGG trinucleotide expansion in the promoter region of the *FMR1* gene. This abnormal CGG trinucleotide expansion can be classified into different allelic forms, including normal allele (5-44 repeats), premutation allele (55-200 repeats), and full mutation allele (>200 repeats) (Dean *et al.*, 2018) (**Fig 31**). Individuals with the premutation allele can develop fragile X-associated tremor/ataxia syndrome (FXTAS) characterized by ataxia, deficits in executive functions, and neurodegenerative (Hagerman *et al.*, 2017). Interestingly, humans with FXTAS frequently develop peripheral neuropathies associated with a high frequency of painful sensations (Berry-Kravis *et al.*, 2007; Brega *et al.*, 2009). FXTAS does not repress FMRP expression but leads to an increase in FMRP mRNA expression (Salcedo-Arellano *et al.*, 2020). In contrast, the full mutation of the allele leads to the hypermethylation of the *FMR1* gene, with the consequent transcriptional silencing and the loss of the FMRP (Penagarikano *et al.*, 2007; Krueger and Bear, 2011). FMRP is an RNA-binding protein, predominantly expressed at the synaptic level, that acts as an inhibitor of local translation in neurons. The loss of this

protein impairs protein synthesis and synaptic plasticity and causes the FXS (Darnell *et al.*, 2011). Due to the X-linked dominant inheritance, the prevalence of the syndrome is lower in women than in men (Hagerman *et al.*, 2017).

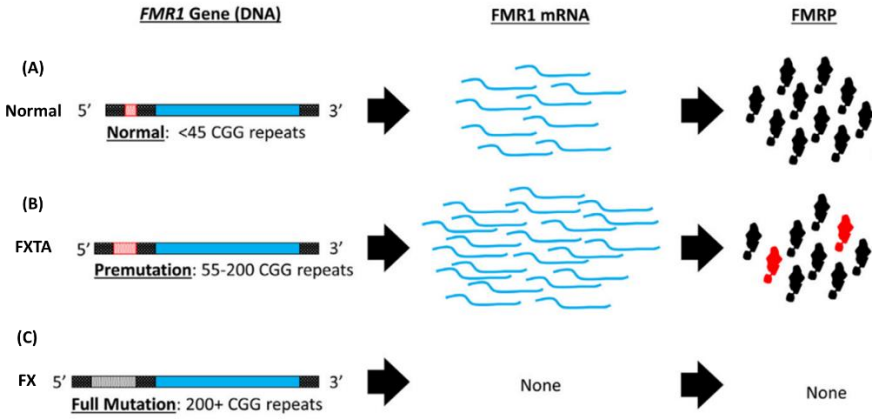

Figure 31. Schematic representation of *FMR1* expression depending on trinucleotide CGG repeat length and its associated clinical phenotype. Within the *FMR1* gene, non-coding 5′ and 3′ regions are indicated with shaded black patterns, whereas the reading frame is indicated in blue. CGG repeat is located in the 5′ untranslated region (red-shaded in A and B, grey-shaded in C to represent hypermethylation of the gene). *FMR1* mRNA transcripts are indicated with curved blue lines. FMRP protein is represented as black shapes and toxic FMRpolyG is represented as red shapes. CGG, cysteine-guanine-guanine trinucleotide; FXTAS, fragile X-associated tremor/ataxia syndrome; FXS, fragile X syndrome. Adapted from (Salcedo-Arellano *et al.*, 2020).

4.3. Pathological aspects in fragile X syndrome

4.3.1 Physical and behavioral alterations

FXS patients present heterogeneous physical and behavioral alterations that can vary considerably within individuals.

Concerning the **physical characteristics**, individuals suffering from this syndrome show a long face with large and prominent ears and a broad forehead (Hagerman *et al.*, 2017). Other physical features include macroorchidism, hyperextensible joints, hypotonia, and mitral valve prolapse, among others (Stone *et al.*, 2021).

Regarding **behavioral abnormalities**, the most prominent neurological phenotype found in FXS is intellectual disability. FXS patients show IQ values between 20 and 70 accompanied by alterations in working and short-term memory, mathematical and visuospatial abilities, and executive functioning (Penagarikano *et al.*, 2007; de Esch *et al.*, 2014). Additional features of FXS include anxiety-like behaviors, epileptic seizures, self-injurious behavior, hypersensitivity to sensory stimulation, and other neurodevelopmental problems, including attention deficits and autistic-like behavior (Hagerman, 2006; Cornish *et al.*, 2008).

4.3.2 Cellular and molecular alterations

Due to the lack of FMRP, several cellular and molecular alterations have been demonstrated in FXS in the central nervous system (T. Wang *et al.*, 2012). FXS patients show an abnormal dendritic spine density and morphology related to alterations in protein synthesis in neurons (Ligsay and Hagerman, 2016). These alterations in the **dendritic spines** structure and/or density have been observed in several brain regions, such as the CA1 region of the hippocampus (Busquets-Garcia *et al.*, 2013), and the cortex (Salcedo-Arellano *et al.*, 2020). Thus, synaptic plasticity deficits in these brain areas could lead to the intellectual disability feature described in the FXS (Nimchinsky *et al.*, 2001).

The altered dendritic spine morphology may underlie disturbances in the synaptic transmission of neuronal circuits in FXS patients. Indeed, **hyperactivation** of the excitatory **mGluR5** has been described in FXS (Bear *et al.*, 2004), and genetic reduction of mGluR5 expression is sufficient to normalize some features of FXS in the *Fmr1* knockout (KO) mouse model (Michalon *et al.*, 2012). In normal conditions, glutamate stimulates mGluR5 to induce local mRNA translation, leading to the synthesis of new proteins that consequently internalize AMPA. This mGluR-dependent mechanism is called long-term depression (mGluR-LTD) (Levenga *et al.*, 2010). However, the uncontrolled translation due to the absence of FMRP in the FXS increases the synthesis of new proteins necessary for the internalization of AMPA and exacerbates the LTD. The supposition that particular FXS symptoms could be explained by excessive mGluR-LTD led to the so-called "**mGluR theory of FXS**" (**Fig. 32**) (Bear *et al.*, 2004; Levenga *et al.*, 2010).

Both the glutamatergic and the GABAergic theories propose an exaggerated mGluR signaling and a decreased GABA signaling, suggesting an excitatory/inhibitory imbalance that could underlie most of the features that characterize the FXS.

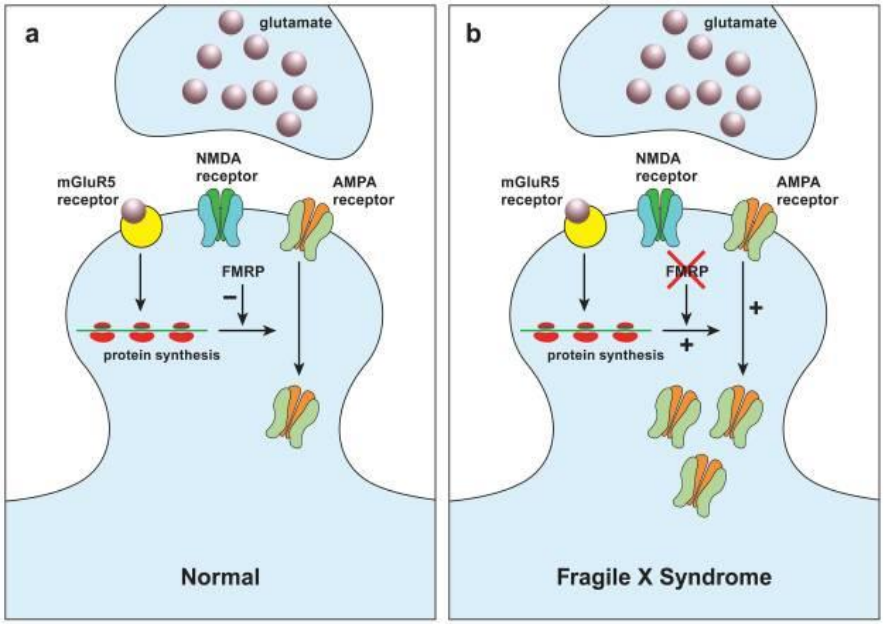

Figure 32. The mGluR theory of fragile X syndrome. a) In normal conditions, stimulation of metabotropic glutamate receptor 5 (mGluR5) by glutamate induces local mRNA translation in the synapse. Local protein synthesis stimulates the internalization of α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid receptors (AMPA), which is essential for long-term depression (LTD). FMRP modulates mGluR activity by negatively regulating translation and reducing the internalization of AMPA receptors. **b)** In fragile X syndrome, the absence of FMRP leads to an excessive protein synthesis that induces AMPA internalization producing an exaggerated mGluR-LTD. NMDA, N-methyl-D-aspartate receptors; FRMP, fragile X mental retardation protein. Extracted from (Levenga *et al.*, 2010).

4.6 Mouse models of fragile X syndrome

Nowadays, several genetically modified mice reproduce some of the most important traits of the fragile X syndrome, including knock-out mouse models of either *Fmr1* or *Fmr2* genes and knock-in mouse models of the CGG repeat expansion (**Table 8**). Preclinical mouse models of fragile X syndrome are also obtained by disrupting the

paralogous *Fxr1* and *Fxr2* genes (Siomi *et al.*, 1995; Bontekoe *et al.*, 2002). These genetic tools are of huge utility for studying the different behavior, cellular and molecular alterations present in this disorder allowing the evaluation of potential novel therapeutic approaches. The most widely used mouse model of FXS is the Fmr1KO mouse, obtained by interrupting the murine *Fmr1* gene (Kooy *et al.*, 1996) that causes the loss of FMRP production. Although the Fmr1KO mice are not representative of the CGG expansion as other mouse models, it keeps the loss of FMRP production (Bakker *et al.*, 1994; Kooy, 2003), recreating the same situation found in human patients.

The behavioral phenotype previously described has been mainly characterized in the Fmr1KO mouse, including the learning and memory impairment (Busquets-Garcia *et al.*, 2013), seizure susceptibility (Michalon *et al.*, 2012), anxiety-like behavior (Jung *et al.*, 2012), and nociceptive desensitization (Price *et al.*, 2007). All these features are sensitive to pharmacological or genetic intervention in the Fmr1KO mouse.

Genetic approach	Mouse model	Modification	References
Knock-out model	Fragile X knockout mice	Frm1 KO	(Bakker *et al.*, 1994)
		Frm2 KO	(Mientjes *et al.*, 2006)
Paralogous genes	FXR1	FXR1 KO	(Siomi *et al.*, 1995)
	FXR2	FXR2 KO	(Bontekoe *et al.*, 2002)
Repeat expansion	Transgenic	$(CGG)_{60}$	(Bontekoe *et al.*, 1997)
		$(CGG)_{43}$	(Lavedan *et al.*, 1997)
		$(CGG)_{97}$	(Lavedan *et al.*, 1998)
	Knock-in	$(CGG)_{98}$	(Bontekoe *et al.*, 2001)
	Transgenic rescue	FMR1 cDNA	(Bakker *et al.*, 2000)
		FMR1 YAC	(Peier *et al.*, 2000)

Table 8. Mouse models of fragile X syndrome. The *Fxr1* and *Fxr2* genes are autosomal homologs 1 and 2 of the *Fmr1* gene. Adapted from (Kooy, 2003; Wijetunge *et al.*, 2013).

4.7 The endocannabinoid system in fragile X syndrome

The ECS is also dysregulated in the absence of FMRP. Some studies have demonstrated an uncoupling of DAGLα from the mGluR5-Homer complex in Fmr1KO mice (Jung *et al.*, 2012; Tang and Alger, 2015) that leads to impaired 2-AG production and subsequently decreased suppression of both GABAergic and glutamatergic transmission (Lutz *et al.*, 2015). Indeed, mGluR5 and CB1R have been shown to have functional crosstalk that regulates a variety of physiological and

pathological conditions (Olmo *et al.*, 2016). These alterations in the ECS seem to participate in the imbalance between excitatory and inhibitory inputs in FXS since targeting this endogenous system alleviates most of the features that characterize this disease. On the one hand, increasing 2-AG signaling through MAGL inhibition normalized synaptic plasticity and behavioral changes in the FXS mouse model (Jung *et al.*, 2012). On the other hand, genetic deletion or pharmacological blockage of CB1R normalized object-recognition memory deficits, susceptibility to audiogenic seizures, altered spine morphology in the CA1 hippocampal region, and the aberrant mGluR5-LTD in Fmr1KO mice (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016). Furthermore, the blockade of CB2R alleviates the anxiety-like phenotype described in Fmr1KO mice (Busquets-Garcia *et al.*, 2013).

OBJECTIVES

General objective

The general objective of this thesis is to study the implication of CB2R in two specific pathological conditions, neuropathic pain and food addiction, to clarify the possible therapeutic interest of this endogenous target. We have focused our attention on exploring the contribution of CB2R in several behavioral pain, emotional and addictive-like manifestations using relevant mouse models.

Objective 1

To explore the mechanisms underlying the pain-resistant phenotype of Fmr1KO mice against the nociceptive and emotional manifestations triggered by persistent nerve damage.

Chapter 1: Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain.

Objective 2

To investigate the participation of the CB2R in the control of palatable food intake and the emotional manifestations associated with food addiction.

Chapter 2: Unraveling the role of CB2 cannabinoid receptor presence to develop resilience or vulnerability to food addiction.

The **Annex** includes two manuscripts related to the implication of CB2R in the development and maintenance of neuropathic pain.

Article 1#

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain.

Ramírez-López Á, Pastor A, de la Torre R, La Porta C, Ozaita A, Cabañero D, Maldonado R.

Eur J Pain. 2021 Feb 22. DOI: 10.1002/ejp.1753

Article 2#

Protective role of neuronal and lymphoid cannabinoid CB2 receptors in neuropathic pain.

Cabañero D, Ramírez-López A, Drews E, Schmöle A, Otte DM, Wawrzczak-Bargiela A, Huerga Encabo H, Kummer S, Ferrer-Montiel A, Przewlocki R, Zimmer A, Maldonado R.

Elife. 2020 Jul 20. DOI: 10.7554/eLife.55582

RESULTS

Chapter #1

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain.

Ramírez-López Á, Pastor A, de la Torre R, La Porta C, Ozaita A, Cabañero D, Maldonado R.

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain.

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1.1. Abstract

Neuropathic pain is a complex condition characterized by sensory, cognitive, and affective symptoms that magnify the perception of pain. The underlying pathogenic mechanisms are largely unknown and there is an urgent need for the development of novel medications. The endocannabinoid system modulates pain perception and drugs targeting the cannabinoid receptor type 2 (CB2R) devoid of psychoactive side effects could emerge as novel analgesics. An interesting model to evaluate the mechanisms underlying resistance to pain is the fragile X mental retardation protein knockout mouse (Fmr1KO), a model of fragile X syndrome that exhibits nociceptive deficits and fails to develop neuropathic pain. A partial sciatic nerve ligation was performed on wild-type (WT) and Fmr1KO mice having (HzCB2 and Fmr1KO-HzCB2, respectively) or not (WT and Fmr1KO mice) a partial deletion of CB2R to investigate the participation of the endocannabinoid system on the pain-resistant phenotype of Fmr1KO mice. Nerve injury induced canonical hypersensitivity in WT and HzCB2 mice, whereas this increased pain sensitivity was absent in Fmr1KO mice. Interestingly, Fmr1KO mice partially lacking CB2R lost this protection against neuropathic pain. Similarly, pain-induced depressive-like behavior was observed in WT, HzCB2, and Fmr1KO-HzCB2 mice, but not in Fmr1KO littermates. Nerve injury evoked different alterations in WT and Fmr1KO mice at spinal and supraspinal levels that correlated with these nociceptive and emotional alterations. This work shows that CB2R is necessary for the protection against

neuropathic pain observed in Fmr1KO mice, raising the interest in targeting this receptor for the treatment of neuropathic pain.

1.2. Introduction

Neuropathic pain is defined as an unpleasant sensory and emotional experience initiated by a lesion or disease of the somatosensory nervous system, mainly associated with spontaneous pain, hyperalgesia, and allodynia (Scholz *et al.*, 2019). Neuropathic pain patients often experience anxiety, depression, and impaired cognitive functions that diminish their life quality (Descalzi *et al.*, 2017). This clinical entity that affects millions of people worldwide (Colloca *et al.*, 2017) has pathogenic mechanisms that remain largely unknown, and current treatments are limited by the lack of efficacy and important side effects (Bouhassira and Attal, 2018). Therefore, there is an urgent need to develop novel therapeutic strategies to improve the quality of life of neuropathic pain patients.

The fragile X mental retardation protein (FMRP), required for the adequate development of neuronal connections, has raised the new interest to clarify pain processing (Busquets-Garcia *et al.*, 2014; Aloisi *et al.*, 2017). The absence of FMRP in humans causes the most common monogenic condition of autistic spectrum disorders, the fragile X syndrome, and a prominent feature of this disorder is self-injurious behavior associated with an alteration of the nociceptive system (Peebles and Price, 2012). Likewise, the preclinical mouse model of fragile X syndrome, a knockout mouse lacking FMRP (Fmr1KO), shows profound deficits in nociceptive sensitization during neuropathic pain

(Price *et al.*, 2007). Therefore, the pain-resistant phenotype of the Fmr1KO mouse represents an appropriate model to investigate the mechanisms underlying these nociceptive deficits and their comorbid manifestations, including cognitive impairment and depressive-like behavior.

The endocannabinoid system is involved in several physiological processes including affective, cognitive, and nociceptive functions (Rácz *et al.*, 2015; Donvito *et al.*, 2018). Cannabinoid receptors type 1 (CB1R) and type 2 (CB2R) are distributed in main central and peripheral nervous system areas involved in pain processing, such as the medial prefrontal cortex (mPFC), amygdala, and spinal cord (La Porta *et al.*, 2015). The activation of both receptors reduces nociception and affective alterations produced by neuropathic pain (Klauke *et al.*, 2014; Rácz *et al.*, 2015). We have previously demonstrated that the pain-resistant phenotype of Fmr1KO mice in a model of inflammatory pain depends on CB1R presence (Busquets-Garcia *et al.*, 2013). However, due to its widespread brain distribution, CB1R activation leads to important psychoactive, motor, and cognitive effects, which represent major limitations for chronic pain treatment (Davis, 2014). Alternative approaches have been developed to overcome this problem by targeting CB2R that is predominantly expressed in peripheral immune cells, although it is also present at low levels in neurons (Shang and Tang, 2017). Therefore, CB2R could be a potential therapeutic target for neuropathic pain treatment avoiding the risk of centrally-mediated side effects.

This study aimed to elucidate the neurobiological mechanisms involved in the pain-resistant phenotype of Fmr1KO mice in order to identify potential pharmacological targets for neuropathic pain. For this purpose, we evaluated the nociceptive, cognitive, and affective manifestations associated with a peripheral nerve injury in Fmr1KO mice partially lacking CB2R to explore the therapeutic interest of this receptor for neuropathic pain.

1.3. Methods

Animals

Taken into account that fragile X syndrome predominantly occurs in male individuals (Razak *et al.*, 2020), all experiments were performed in male mice between 8 and 20 weeks of age. WT ($Fmr1^{+/y}$, $Cnr2^{+/+}$), Fmr1KO ($Fmr1^{-/y}$, $Cnr2^{+/+}$), WT heterozygous for CB2R (HzCB2) ($Fmr1^{+/y}$, $Cnr2^{+/-}$) and Fmr1KO heterozygous for CB2R (Fmr1KO-HzCB2) ($Fmr1^{-/y}$, $Cnr2^{+/-}$) littermates in C57BL/6J genetic background were used. The behavioral experiments were conducted in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). Mice were group-housed (2-4 animals) and maintained in a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%) environment. Food and water were available ad libitum and mice were handled during the light phase of a 12 h light/dark cycle (light on at 8:00 a.m., light off at 8:00 p.m.). All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive

(2010/63/EU). All the experiments were performed under blind and randomized conditions.

Neuropathic pain induction

Mice underwent a partial sciatic nerve ligation (PSNL) at mid-thigh level to induce neuropathic pain, as previously described (Malmberg and Basbaum, 1998) with minor modifications. Briefly, mice were anesthetized with isoflurane (induction, 5% V/V; surgery, 2% V/V) in oxygen and the sciatic nerve was exposed at the level of the mid-thigh of the right hind leg. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the cranial side of the sciatic nerve using a 9–0 non-absorbable virgin silk suture (Alcon Cusí SA, Barcelona, Spain) and leaving the rest of the nerve untouched. The muscle was then stitched with 6-0 silk (Alcon Cusí), and the skin incision was closed with wound clips. Sham-operated mice underwent the same surgical procedure except that the sciatic nerve was not ligated.

Nociception

Sensitivity to mechanical and heat stimuli was used as nociceptive measures of neuropathic pain. Ipsilateral and contralateral hind paw withdrawal thresholds were evaluated the day before, 3, 7, and 14 days after the nerve injury. Mechanical allodynia was quantified by measuring the withdrawal response to von Frey filament stimulation through the up-down paradigm, as previously reported (Chaplan *et al.*, 1994). Filaments equivalent to 0.04, 0.07, 0.16, 0.4, 0.6, 1, and 2 g were used, applying first the 0.4 g filament and increasing or decreasing the strength according to the response. The filaments were bent and held

for 5 s against the surface of the hind paws. Heat sensitivity was assessed by recording the hind paw withdrawal latency in response to radiant heat applied with the plantar test apparatus (Ugo Basile, Varese, Italy) as previously reported (Hargreaves *et al.*, 1988). Clear paw withdrawal, shaking, or licking was considered a nociceptive response.

Cognitive performance

The novel object-recognition test was performed on day 12 after the surgery as previously described (Puighermanal *et al.*, 2009). Briefly, mice were habituated to a V-shaped maze for 9 min on day 1. The following day, mice were introduced in the maze where 2 identical objects (familiar objects) were presented in the extremes of the maze. For the memory test performed on the third day, 1 of the familiar objects was replaced with a new object (novel object), and the total time spent exploring each of the 2 objects (novel and familiar) was measured. Object exploration was defined as the orientation of the nose towards the object at a distance of <1 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time exploring both objects: (DI = (Tn − Tf)/(Tn + Tf)). A higher discrimination index is considered to reflect greater memory retention for the familiar object. Mice that explored <10 s both objects were excluded from the analysis.

Depressive-like behavior

Depressive-like behavior was evaluated 19 days after the surgery using the forced swimming test (Porsolt and Bertin, 1977). Briefly, mice were individually placed into a glass cylinder (17.5 x 12.5 cm) filled 15 cm high with water (22 ± 1°C). Mice were subjected to forced swimming for 6 min and the total duration of immobility, disregarding small hind limb movements to keep the head above water, was measured during the last 4 min when mice show a sufficiently stable level of immobility.

Endocannabinoid quantification

Animals were sacrificed at the end of the experimental protocol and L3-L5 ipsilateral spinal cord dorsal horns were freshly dissected. Samples were rapidly frozen and stored at -80°C. The quantification of endocannabinoids and related compounds was based on the methodology previously described in plasma (Pastor *et al.*, 2014), adapted for the extraction of endocannabinoids from spinal tissue. The following endocannabinoids and related compounds were quantified: 2-arachidonoyl glycerol (2-AG), N-arachidonoylethanolamine (AEA), palmitoylethanolamide (PEA), and oleoylethanolamine (OEA). Frozen spinal cords (4 ± 1 mg) of mice were placed in a 1 ml Wheaton glass homogenizer and spiked with 25 µl of a mix of deuterated internal standards dissolved in acetonitrile. The mix contained 5 ng/ml of each compound. All internal standards were purchased from Cayman Chemical (Ann Harbor). Tissues were homogenized on ice with 700 µl a mixture of 50 mM Tris-HCl buffer (pH 7.4): methanol (1:1) and the homogenates were transferred to 12 ml glass tubes. The homogenizer was washed twice with 0.9 ml of the same mixture and the contents

were combined into the tube giving an approximate volume of 2.5 ml of homogenate. The homogenates were kept on ice until organic extraction to minimize the ex-vivo generation of endocannabinoids. Next, homogenates were extracted with 5 ml chloroform over 20 min by placing the tubes in a rocking mixer. Tubes were centrifuged at 1700 g over 5 min at room temperature. The lower organic phase was transferred to clean glass tubes, evaporated under a stream of nitrogen in a 39 °C water bath, and extracts were reconstituted in 100 µl of mixture water: acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to high-performance liquid chromatography vials with glass micro-vials. Endocannabinoids were separated using an Agilent 6410 triple quadrupole Liquid-Chromatograph equipped with a 1200 series binary pump, a column oven, and a cooled autosampler (4 °C). Chromatographic separation was carried out with a Waters C18-CSH column (3.1×100 mm, 1.8 µm particle size) maintained at 40°C with a mobile phase flow rate of 0.4 ml/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. Endocannabinoids and related compounds were separated by gradient chromatography. The ion source was operated in the positive electrospray mode. The selective reaction monitoring mode was used for the analysis. Quantification was done by isotope dilution with the response of the deuterated internal standards and data were expressed as a percentage of the control group (WT sham).

Gene expression analysis

Animals were sacrificed at the end of the experimental protocol and mPFC, amygdala (left and right, separately), and spinal ipsilateral dorsal

horns were freshly dissected. The samples were rapidly placed in individual tubes and stored at -80°C. Total RNA was isolated from frozen samples with RNeasy Micro kit (74004, Qiagen, Stokach, Germany) and subsequently reverse-transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time-Polymerase Chain Reaction (RT-PCR) was carried out in triplicate with a QuantStudio 12K Flex RT-PCR System (4471134, Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (04707516001, Roche, Basel, Switzerland). The expression of the following genes was analyzed: nuclear factor NF-Kappa-B P65 subunit (*Rela*), glutamate metabotropic receptor 5 (*Grm5*), homer scaffolding protein 1a (*Homer1a*), and peroxisome proliferator-activated receptor alpha (*Ppara*). Levels of the target genes were normalized against the housekeeping gene beta-2-microglobulin (*B2m*) and compared using the ΔΔCt method (Livak and Schmittgen, 2001). The following specific primers were used: 5'-CTTCCTCAGCCATGGTACCTCT-3' (*Rela* forward); 5'-CAAGTCTTCATCAGCATCAAACTG-3' (*Rela* reverse), 5'-GTCCTGGCCCACTGACGA-3' (*Grm5* forward); 5'-GGTCACCCCATCGAAGATAC-3' (*Grm5* reverse), 5'-GGGAGGATGGAGACACAGC-3' (*Homer1a* forward); 5'-CGGTCCGTCCCTTTTTCCTT-3' (*Homer1a* reverse), 5'-AGAGGGCTGAGCGTAGGTAA-3' (*Ppara* forward); 5'-ATTGGGCCGGTTAAGACCAG-3' (*Ppara* reverse), 5'-TTCTGGTGCTTGTCTCACTGA-3' (*B2m* forward); 5'-CAGTATGTTCGGCTTCCCATTC-3' (*B2m* reverse).

Statistics

All the data were first subjected to a Shapiro-Wilk test of normality. The time course of nociceptive thresholds was analyzed using a linear mixed model with three factors (surgery, genotype, time, and their interactions) considering the presence of non-gaussian distribution in some of the experimental days. Bonferroni *post hoc* analysis was performed when pertinent. Long-term memory and endocannabinoid quantification and RT-PCR data were analyzed with a two-way ANOVA (genotype, surgery) followed by Bonferroni *post hoc*. Depressive-like behavior and endocannabinoid quantification and RT-PCR data were analyzed with a Kruskal-Wallis followed by U Mann Whitney with Bonferroni adjustment for multiple comparisons taken into account that these data did not follow a normal distribution. A probability of 0.05 or less was considered statistically significant. Detailed statistical analysis is presented in Supplementary Tables S1-S4.

1.4. Results

Role of CB2R in the nociceptive manifestations of neuropathic pain in Fmr1KO mice

Nociceptive sensitivity to mechanical and heat stimulation was evaluated under basal conditions and 3, 7, and 14 days after PSNL or sham surgery (**Fig. 1**).

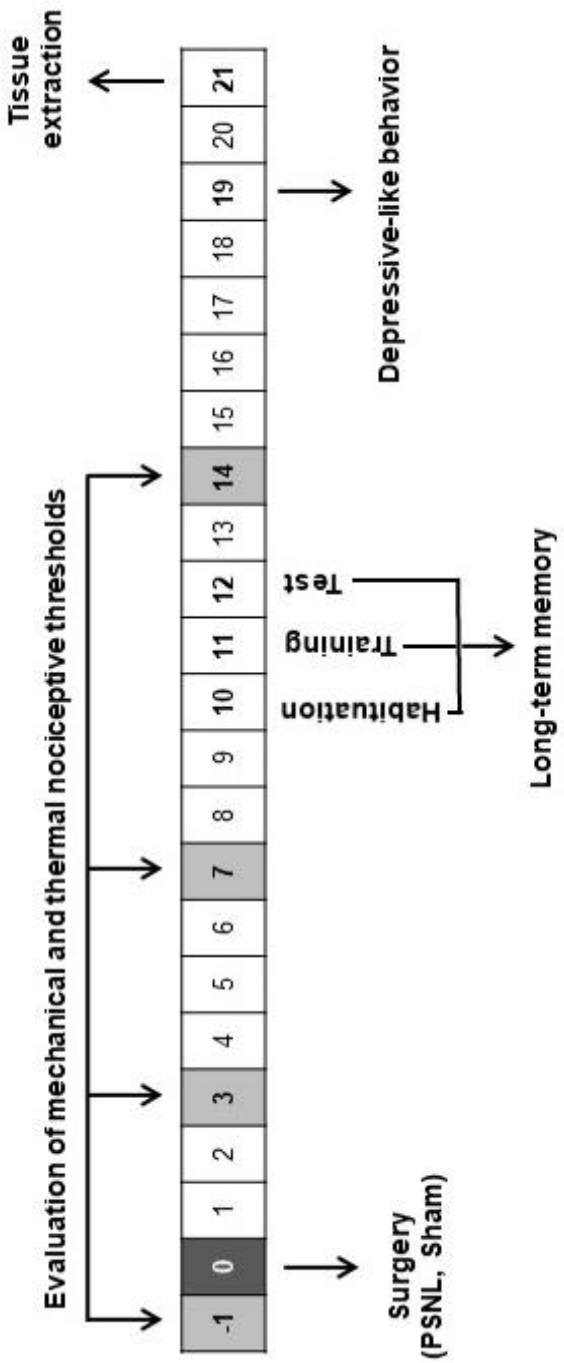


Figure 1. Experimental procedure. Baseline mechanical and heat nociceptive thresholds were measured the day before PSNL or sham surgery. Mechanical and heat nociception were assessed again at days 3, 7 and 14 after the surgery. Cognitive performance and affective behavior were also evaluated under sham and neuropathic pain conditions. Long-term memory was evaluated at day 12 and depressive-like behavior was assessed at day 19 after the surgery. Mice were sacrificed 21 days after the surgery and tissue collection was performed for molecular analysis.

Mechanical allodynia

The von Frey test was used to assess sensitivity to mechanical stimuli in sham and neuropathic pain conditions. All mice showed similar nociceptive thresholds in naïve conditions in both ipsilateral and contralateral paws (**Fig. 2A**). After surgery, nerve-injured animals developed mechanical allodynia, as revealed by significant differences between ipsilateral and contralateral paw sensitivity. As expected, Fmr1KO mice revealed decreased mechanical allodynia following nerve injury when compared to WT mice. Interestingly, the WT mice phenotype on the mechanical nociceptive responses was rescued when CB2R was partially removed in Fmr1KO mice (**Fig. 1A**). No significant differences in mechanical thresholds following sham surgery were observed between WT and the other genotypes (**Fig. 2A**). Therefore, the deletion of the Fmr1 gene decreases the mechanical allodynia associated with the nerve injury and CB2R is involved in this phenotype.

Heat hyperalgesia

Sensitivity to thermal stimuli in the plantar test was also used as a nociceptive measure of neuropathic pain. All mice showed similar withdrawal latencies of both ipsilateral and contralateral paws in naïve conditions (**Fig. 2B**). The nerve injury induced heat hyperalgesia in all genotypes in comparison to the contralateral paw, but this nociceptive behavior was significantly attenuated on Fmr1KO mice. These mutants showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery compared to WT littermates. When CB2R was partially removed from Fmr1KO mice (Fmr1KO-HzCB2 mutants), thermal

hypersensitivity was similar to WT mice (**Fig. 2B**). Sham surgery did not alter the plantar withdrawal latencies of any group (**Fig. 2B**). Thus, the deletion of the Fmr1 gene decreases the heat hypersensitivity associated with the nerve injury and CB2R is also involved in this phenotype.

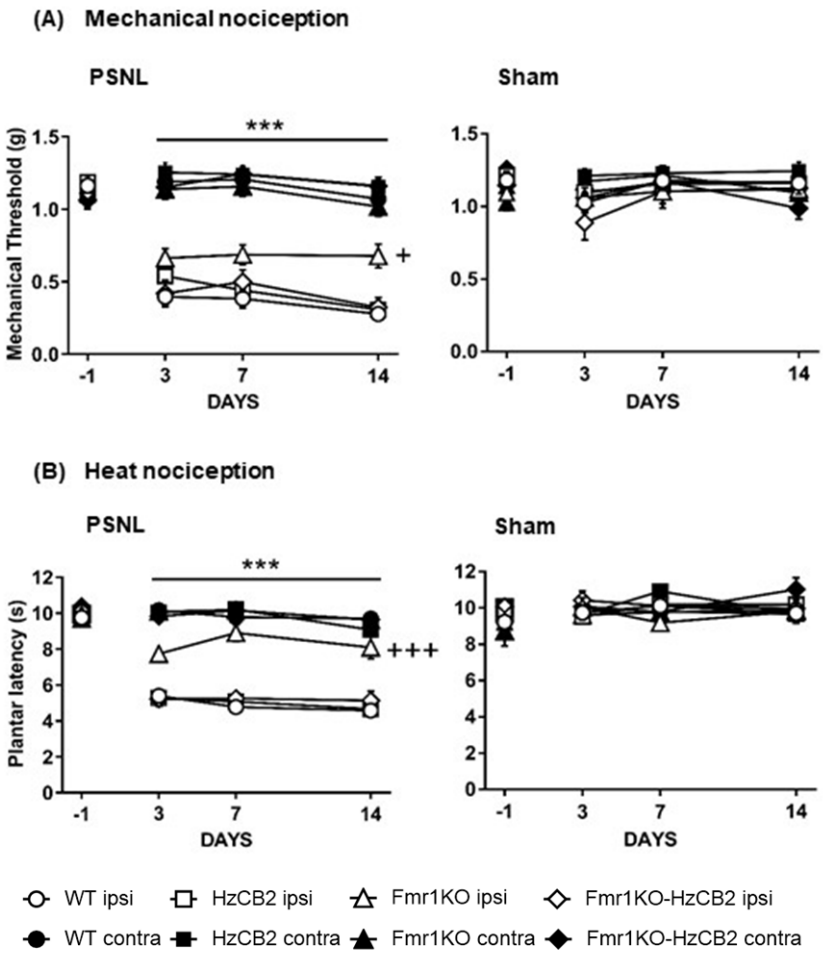


Fig. 2. Nociceptive sensitivity to mechanical and heat stimulation in sham and neuropathic pain conditions. Mice were tested on the ipsilateral and contralateral paws to evaluate mechanical allodynia (mechanical thresholds in grams) in the von Frey test and heat hyperalgesia (plantar latency in seconds) in

the plantar test under basal conditions and on day 3, 7, and 14 after PSNL or sham surgery. **(A)** The development of mechanical allodynia after nerve injury in mice was demonstrated by significant differences between contralateral and ipsilateral paw sensitivity, which was significantly attenuated on the ipsilateral paw of Fmr1KO mice. No differences in mechanical thresholds following sham surgery were observed between the different genotypes. **(B)** Heat hyperalgesia after nerve injury was also confirmed by significant differences between contralateral and ipsilateral paw withdrawal latencies. Fmr1KO mice also showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery in comparison to WT mice. No differences in heat hypersensitivity following sham surgery were observed between the different genotypes. Data are expressed as mean ± SEM (n = 13-19 per group). ***p<0.001 between contralateral and ipsilateral paws; +p<0.05, +++p<0.001 vs. WT ipsilateral paw (Linear mixed model, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S1.

Role of CB2R in memory and emotional alterations associated with neuropathic pain in Fmr1KO mice

Cognitive performance and depressive-like behavior were evaluated under sham and neuropathic pain conditions 12 and 19 days after the surgery, respectively (**Fig. 1**).

Cognitive performance

The novel object recognition test was used to assess long-term memory after the induction of neuropathic pain or sham surgery. Long-term memory impairment was revealed in WT and HzCB2 mice exposed to PSNL by a decrease in the discrimination index in comparison to sham littermates, whereas no significant effect was observed in Fmr1KO and Fmr1KO-HzCB2 mice. These mutants already showed a low discrimination index compared to WT mice regardless of the surgery

(**Fig. 3A**). Hence, both nerve injury and lack of Fmr1 gene produce cognitive impairment, that is not modified by the partial deletion of CB2R.

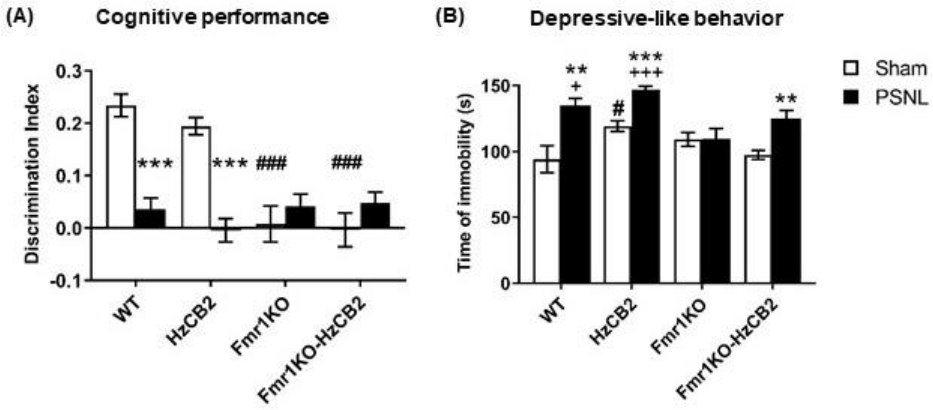


Fig. 3. Cognitive and emotional behaviors in mice exposed to sham or PSNL surgery. Long-term memory impairment (discrimination index in the novel recognition test) and depressive-like behavior (time of immobility in seconds in the forced swimming test) were evaluated 12 and 19 days after the induction of the neuropathy, respectively. **(A)** Nerve injury impaired memory significantly in WT and HzCB2 mice compared to sham mice, whereas Fmr1KO and Fmr1KO-HzCB2 mice show a low discrimination index regardless of the surgery. **(B)** PSNL significantly increased the depressive-like behavior in WT, HzCB2, and Fmr1KO-HzCB2 mice, but not in Fmr1KO mice, as indicated by the time of immobility. HzCB2 mice showed a pro-depressive phenotype under sham conditions in comparison to WT sham animals. Data are expressed as mean ± SEM (n = 11-22 per group). Novel object recognition test: ***p<0.001 vs. Sham mice of each genotype; ###p<0.001 vs. WT sham (ANOVA, Bonferroni test). Forced swimming test: *p<0.05, **p<0.01, ***p<0.001 vs. Sham mice of each genotype; #p<0.05 vs. WT sham; +p<0.05, +++p<0.001 vs. Fmr1KO PSNL mice. (Kruskal-Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S2.

Depressive-like behavior

The forced swimming test was used to assess depressive-like behavior associated with neuropathic pain. As expected, nerve injury induced depressive-like behavior revealed by a significant increase in the time of immobility of WT and HzCB2 animals in comparison to sham-operated mice. Interestingly, HzCB2 mutants subjected to the sham surgery showed increased depressive-like behavior when compared to WT sham mice, suggesting a possible anti-depressive effect of CB2R. On the other hand, Fmr1KO mice did not develop depressive-like behavior associated with the PSNL surgery and showed different responses to nerve-injured WT and HzCB2 mice. However, the depressive-like behavior was rescued in Fmr1KO mutants partially lacking CB2R (**Fig. 3B**). Therefore, the inhibition of depressive-like behavior in Fmr1KO mice depends on CB2R presence, which also modifies this behavior under basal conditions.

Effects of nerve injury on modulating the endocannabinoid tone at spinal cord level

To explore the possible mechanisms underlying the effects of Fmr1 deletion on the nociceptive manifestations of neuropathic pain, we evaluated the levels of endocannabinoids and related lipids in the spinal cord dorsal horn of WT and Fmr1KO mice. 2-arachidonoylglycerol (2-AG) and N-arachidonoyl-ethanolamine (AEA) are the main endogenous ligands of CB1RR and CB2RR, whereas palmitoylethanolamide (PEA) and oleoylethanolamid (OEA) belong to the N-acylethanolamines family and display anti-inflammatory properties that could participate in the protective phenotype of

Fmr1KO mice mainly through peroxisome proliferator-activated receptors (PPARs) (Di Marzo, 2018). Nerve injury did not change the expression of 2-AG, AEA, PEA, or OEA in WT mice compared to sham littermates at the spinal level (**Fig. 4A-B**). In contrast, nerve-injured Fmr1KO mice showed increased levels of PEA and OEA, but not 2-AG or AEA when compared to sham mutants (**Fig. 4C-D**). Sham-operated Fmr1KO mice did not show significant alterations in any of these endocannabinoids in comparison to control WT mice (**Fig. 4A-D**). These findings reveal an association between N-acylethanolamines levels and the nociceptive changes observed in Fmr1KO mice.

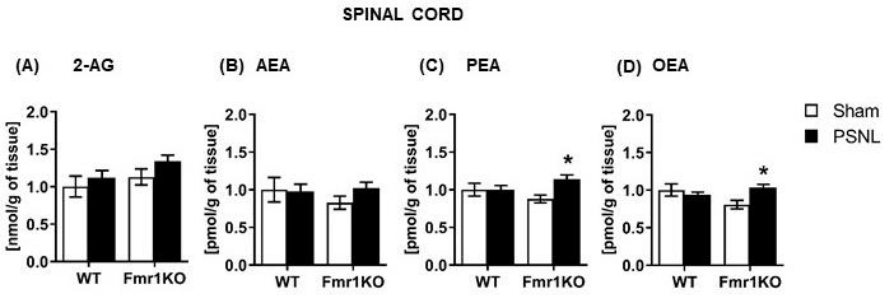


Fig. 4. Quantification of spinal endocannabinoid levels of WT and Fmr1KO mice 21 days after sham or PSNL surgery. High-performance liquid chromatography was used to assess the concentration of 2-AG (nmol/g) **(A)**, AEA (pmol/g) **(B)**, PEA (pmol/g) **(C)** and OEA (pmol/g) **(D)** in the spinal dorsal horn ipsilateral to the site of injury of WT and Fmr1KO mice after a sham of PSNL surgery. Nerve-injured Fmr1KO mice showed upregulated spinal levels of PEA and OEA compared to sham mutants, while 2-AG and AEA spinal levels were no altered. Data are expressed as mean ± SEM (n = 8-11 per group). *p<0.05 vs. Fmr1KO sham mice (ANOVA, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S3.

Molecular changes in somatosensory pain-related areas of nerve-injured WT and Fmr1KO mice

RT-PCR analysis was used to evaluate gene expression levels of pain-related proteins in the spinal cord dorsal horn, mPFC, right and left amygdalae of WT and Fmr1KO mice 21 days after nerve injury.

N-acylethanolamines signaling pathway

PPARs are activated by ligands of different chemical structures, including the endocannabinoid-like compounds PEA and OEA (O'Sullivan, 2016). Considering the increased levels of PEA and OEA in the spinal cord of nerve-injured Fmr1KO mice, we evaluated *Ppara* (PPARα encoding gene) expression in spinal cord and brain areas involved in pain and emotional processing.

WT and Fmr1KO animals did not reveal significant alterations of *Ppara* expression in the spinal cord dorsal horn, although a trend to enhance *Ppara* levels was observed after nerve injury in Fmr1KO mice (**Fig. 5A**). However, nerve-injured Fmr1KO mice showed enhanced *Ppara* expression in the mPFC when compared to sham littermates or nerve-injured WT mice (**Fig. 5B**). In the right amygdala, a global decrease of *Ppara* expression was revealed in Fmr1KO animals compared to WT mice (**Fig. 5C**).

Additionally, PPARα exerts anti-inflammatory effects by modulating the activity of several pro-inflammatory transcription factors, including the nuclear factor-Kappa B (NF-κB) (Rakhshandehroo *et al.*, 2010). NF-κB promotes the expression of genes that participate in the sensitization processes by encoding pro-inflammatory mediators such

as cytokines and brain-derived neurotrophic factor (Grace *et al.*, 2014). The expression of *Rela* (NF-κB encoding gene) was not modified in the spinal cord dorsal horn and right amygdala of WT and Fmr1KO mice regardless of the surgery (**Fig. 5A, C**). In contrast, nerve-injured Fmr1KO animals revealed increased levels of *Rela* expression in the mPFC in comparison to sham littermates and nerve-injury WT mice (**Fig. 5B**), whereas this gene expression was diminished in the left amygdala of Fmr1KO after nerve injury (**Fig. 5D**).

Glutamate signaling

An altered glutamate transmission mainly due to modifications in the metabotropic glutamate receptor 5 (mGluR5) has been implicated in the pathophysiological processes leading to chronic pain and associated affective states (Chung *et al.*, 2017). mGluR5 forms a complex with the Homer Scaffolding Protein 1a (HOMER1a) that plays an important role in glutamate-mediated cellular signaling and nociception (Obara *et al.*, 2013b). We have evaluated the expression levels of the gene coding for mGluR5 protein (*Grm5*) and the HOMER1A encoding gene (*Homer1a*). *Grm5* gene expression was not altered in the spinal cord and left amygdala of WT and mutant mice with or without nerve ligation (**Fig. 5A, D**). However, sham and nerve-injured Fmr1KO mice showed downregulated mRNA levels of *Grm5* in the mPFC compared to the respective WT groups (**Fig. 5B**). On the other hand, WT mice showed increased levels of *Grm5* expression after nerve injury in the right amygdala when compared to sham littermates and nerve-injured Fmr1KO mice (**Fig. 5C-D**). The expression of *Homer1a* was not modified in the spinal cord dorsal horn nor right amygdala of

WT and Fmr1KO mice regardless of the surgery (**Fig. 5A, C**). In the mPFC, a global decrease of *Homer1a* expression was revealed in Fmr1KO animals in comparison to WT mice (**Fig. 5B**). However, *Homer1a* expression was decreased in the left amygdala after PSNL surgery in Fmr1KO mice compared to nerve-injured WT animals (**Fig. 5D**).

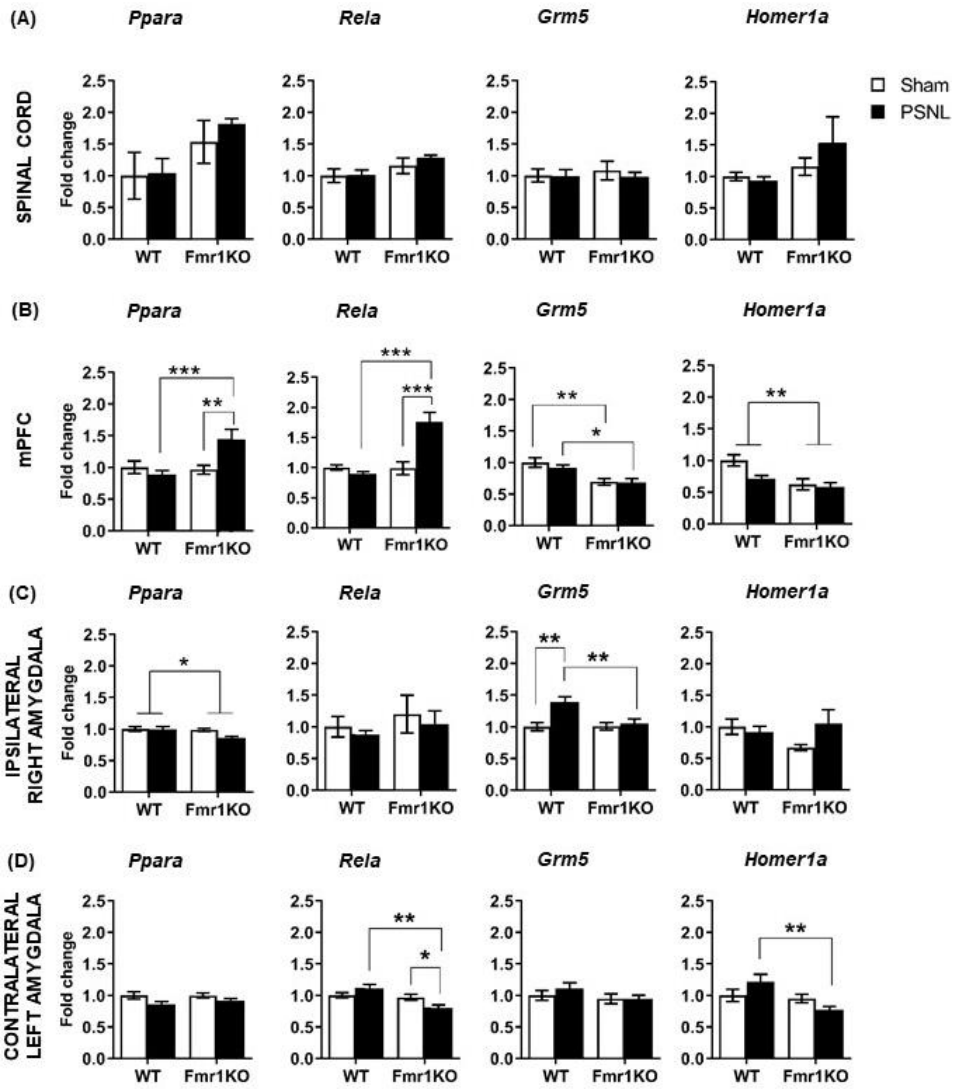


Fig. 5. Molecular changes in somatosensory pain-related areas of WT and Fmr1KO mice 21 days after sham or PSNL surgery. RT-PCR analysis of *Ppara, Rela, Grm5, and Homer1a* gene expression in the spinal ipsilateral dorsal horn, mPFC, right and left amygdala were measured by using the ΔΔCt method. Expression of all these targets was first standardized by expression of B2M and expressed relative to the data taken from the WT sham control group. **(A)** In the spinal cord, RT-PCR analysis of mRNA *Ppara, Rela, Grm5, and Homer1a* expression revealed no significant differences among genotypes. **(B)** In the mPFC, nerve-injured Fmr1KO mice showed upregulated levels of *Ppara* and *Rela* expression compared to WT PSNL and Fmr1KO sham mice, while *Grm5* expression was reduced in mutants. **(C)** In the right amygdala, WT mice with neuropathy exhibited increased levels of *Grm5* expression in comparison to sham littermates and nerve-injured Fmr1KO mice. **(D)** In the left amygdala, Fmr1KO mice after PSNL surgery showed decreased expression of *Rela* and *Homer1a* compared to nerve-injured WT animals. Data are expressed as mean ± SEM (n = 6-10 per group). *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Bonferroni test; Kruskal-Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S4.

Correlations between gene expression and the time of immobility in the water maze

In order to provide additional findings in support of the role of the different brain areas on the effect of Fmr1 gene promoting depressive-like behavior after nerve injury, we evaluated the correlations between gene expression and immobility time of WT and Fmr1KO mice regardless of the type of surgery. Elevated time of immobility in the water maze was positively correlated with the expression in the left amygdala (contralateral to the site of injury) of Rela (r = 0.567; p = 0.007), Grm5 (r = 0.507; p = 0.016) and Homer1a (r = 0.596; p = 0.003), but not with Ppara expression (r = 0.007; p = 0.976) (Fig. 6A-D). These

correlations and the observed decreased gene expression of Homer1a and Rela in the left amygdala of nerve-injured Fmr1KO mice (Fig. 5D) suggest a possible role of the left amygdala in the protective phenotype of Fmr1KO mice against the nociceptive and emotional manifestations of neuropathic pain. No association was revealed between time of immobility and mPFC expression of Ppara (r = -0.067; p = 0.761), Rela (r = 0.256; p = 0.227), Grm5 (r = 0.163; p = 0.406) and Homera1 (r = -0.041; p = 0.834). Similarly, no significant associations were revealed in the right amygdala for Ppara (r = 0.088; p = 0.697), Rela (r = 0.162; p = 0.471), Grm5 (r = 0.357; p = 0.103) and Homera1 (r = -0.280; p = 0.207) expression (data not shown).

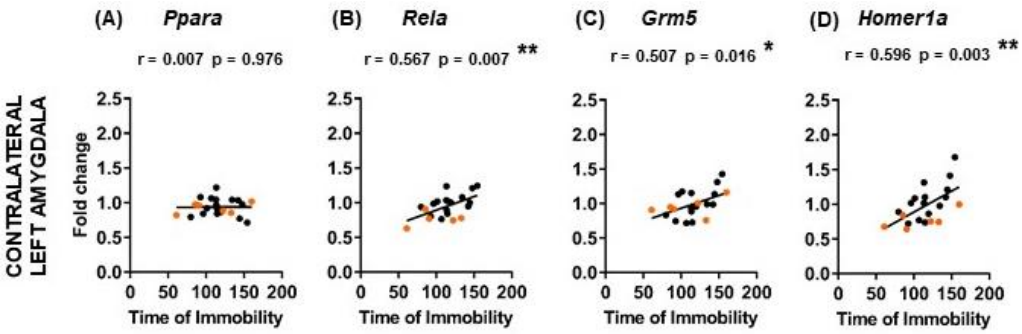


Figure 6. Correlations between gene expression in the left amygdala and depressive-like behavior of WT and Fmr1KO mice 21 days after sham or PSNL surgery. Correlation between the time of immobility in the forced swimming test and the gene expression of *Ppara* **(A)**, *Rela* **(B)**, *Grm5* **(C),** *and Homer1a* **(D)** in the left amygdala of WT and Fmr1KO mice. *Rela, Grm5, and Homer1a* gene expression in the left amygdala, but no *Ppara*, showed significant positive correlations with depressive-like behaviors. Orange dots represent PSNL Fmr1KO mice. Data are expressed as mean (n = 5-7 per group). *p<0.05, **p<0.01 (Pearson Correlation).

Supplementary material

Supplementary table S1. Detailed statistical evaluation for Fig. 1.

Tests of normality

Von-Frey PSNL	**Shapiro-Wilk**
Baseline	0.005
Day 3	0.000
Day 7	0.000
Day 14	0.000

Von-Frey sham	**Shapiro-Wilk**
Baseline	0.000
Day 3	0.000
Day 7	0.000
Day 14	0.000

Plantar PSNL	**Shapiro-Wilk**
Baseline	0.478
Day 3	0.001
Day 7	0.000
Day 14	0.003

Plantar sham	**Shapiro-Wilk**
Baseline	0.824
Day 3	0.003
Day 7	0.064
Day 14	0.038

Linear mixed model with three factors for nociceptive thresholds. Only when F was significant, Linear mixed model was followed by Bonferroni *post hoc.*

Between genotypes (Von-Frey PSNL)	**Pressure**
Genotype	F (3, 520.922) = 3.315; p<0.05
Paw	F (1, 520.922) = 457.620; p<0.001
Genotype x Paw	F (3, 520.922) = 9.419; p < 0.001
Time	F (3, 260.160) = 58.798; p<0.001
Time x Genotype	F (9, 260.160) = 1.241; p = 0.270
Time x Paw	F (3, 260.160) = 77.498; p<0.001
Time x Genotype x Paw	F (9, 260.160) = 1.569; p = 0.125

Between genotypes (Von-Frey sham)	**Pressure**
Genotype	F (3, 498.569) = 4.911; p<0.01
Paw	F (1, 498.569) = 1.972; p = 0.161
Genotype x Paw	F (3, 498.569) = 5.108; p = 0.741
Time	F (3, 245.633) = 2.394; p = 0.069
Time x Genotype	F (9, 245.633) = 1.258; p = 0.261
Time x Paw	F (3, 245.633) = 1.774; p = 0.153
Time x Genotype x Paw	F (9, 245.633) = 0.527; p = 0.854
Between genotypes (Plantar PSNL)	**Heat**
Genotype	F (3, 526.262) = 23.258; p<0.001
Paw	F (1, 526.262) = 617.584; p<0.001
Genotype x Paw	F (3, 526.262) = 23.049; p<0.001
Time	F (3, 255.117) = 94.555; p<0.001
Time x Genotype	F (9, 255.117) = 5.113; p<0.001
Time x Paw	F (3, 255.117) = 84.631; p<0.001
Time x Genotype x Paw	F (9, 255.117) = 2.575; p<0.01

Between genotypes (Plantar sham)	**Heat**
Genotype	F (3, 495.040) = 3.534; p < 0.05
Paw	F (1, 495.040) = 0.240; p = 0.625
Genotype x Paw	F (3, 495.040) = 0.164; p = 0.920
Time	F (3, 237.012) = 1.374; p = 0.251
Time x Genotype	F (9, 237.012) = 1.218; p = 0.284
Time x Paw	F (3, 237.012) = 0.564; p = 0.652
Time x Genotype x Paw	F (9, 237.012) = 0.693; p = 0.715

Supplementary table S2. Detailed statistical evaluation for Fig. 2.

Tests of normality

Novel object recognition test	**Shapiro-Wilk**
WT	0.623
Fmr1KO	0.945
HzCB2	0.119
Fmr1KO-HzCB2	0.227

Forced swimming test	**Shapiro-Wilk**
WT	0.002
Fmr1KO	0.053
HzCB2	0.171
Fmr1KO-HzCB2	0.017

Kruskal-Wallis for emotional manifestations of neuropathic pain. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

Among 8 groups	**Time of immobility**
Time of immobility	H (7) = 40.141, p<0.001

ANOVA model with two factors for cognitive manifestations of neuropathic pain. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

Among 8 groups	**Discrimination Index**
Genotype	F (3, 124) = 10.037; p<0.001
Surgery	F (1, 124) = 15.915; p<0.001
Genotype x Surgery	F (3, 124) = 15.891; p<0.001

Supplementary table S3. Detailed statistical evaluation for Fig. 3.

Tests of normality

2-AG	**Shapiro-Wilk**
WT	0.729
Fmr1KO	0.073
AEA	
WT	0.388
Fmr1KO	0.598
PEA	
WT	0.078
Fmr1KO	0.663
OEA	
WT	0.328
Fmr1KO	0.885

ANOVA model with two factors for High-performance liquid chromatography data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

2-AG	
Genotype	F (1, 34) = 2.701; p = 0.109
Surgery	F (1, 34) = 2.456; p = 0.126
Genotype x Surgery	F (1, 34) = 0.191; p = 0.665
AEA	
Genotype	F (1, 34) = 0.351; p = 0.558
Surgery	F (1, 34) = 0.641; p = 0.429
Genotype x Surgery	F (1, 34) = 1.063; p = 0.310
PEA	
Genotype	F (1, 34) = 0.0.29; p = 0.865
Surgery	F (1, 34) = 4.539; p = 0.040
Genotype x Surgery	F (1, 34) = 4.602; p<0.05
OEA	
Genotype	F (1, 34) = 0.812; p = 0.374
Surgery	F (1, 34) = 2.253; p = 0.143
Genotype x Surgery	F (1, 34) = 15.891; p<0.05

Supplementary table S4. Detailed statistical evaluation for Fig. 4.

Tests of normality

RIGHT AMYGDALA

Ppara	**Shapiro-Wilk**
WT	0.813
Fmr1KO	0.196
Rela	
WT	0.654
Fmr1KO	0.346
Grm5	
WT	0.041
Fmr1KO	0.768
Homer1a	
WT	0.553
Fmr1KO	0.081

SPINAL CORD

Ppara	**Shapiro-Wilk**
WT	0.023
Fmr1KO	0.005
Rela	
WT	0.138
Fmr1KO	0.034
Grm5	
WT	0.020
Fmr1KO	0.013
Homer1a	
WT	0.432
Fmr1KO	0.007

LEFT AMYGDALA

Ppara	**Shapiro-Wilk**
WT	0.865
Fmr1KO	0.339
Rela	
WT	0.281
Fmr1KO	0.398
Grm5	
WT	0.906
Fmr1KO	0.484
Homer1a	
WT	0.799
Fmr1KO	0.093

mPFC

Ppara	***Shapiro-Wilk***
WT	*0.876*
Fmr1KO	*0.700*
Rela	
WT	*0.001*
Fmr1KO	*0.017*
Grm5	
WT	*0.803*
Fmr1KO	*0.700*
Homer1a	
WT	*0.165*
Fmr1KO	*0.001*

ANOVA model with two factors for RT-PCR data. Only when F was significant, ANOVA was followed by Bonferroni *post hoc.*

mPFC

Ppara	
Genotype	F (1, 26) = 7.117; p<0.05
Surgery	F (1, 26) = 3.500; p = 0.073
Genotype x Surgery	F (1, 26) = 9.287; p<0.01
Rela	
Genotype	F (1, 34) = 22.377; p<0.001
Surgery	F (1, 34) = 13.576; p<0.01
Genotype x Surgery	F (1, 34) = 23.729; p<0.001

Homer1a	
Genotype	F (1, 31) = 10.292; p<0.01
Surgery	F (1, 31) = 4.410; p<0.05
Genotype x Surgery	F (1, 31) = 2.486; p = 0.125

LEFT AMYGDALA

Ppara	
Genotype	F (1, 20) = 0.470; p = 0.501
Surgery	F (1, 20) = 6.564; p<0.05
Genotype x Surgery	F (1, 20) = 0.560; p = 0.463
Rela	
Genotype	F (1, 19) = 12.907; p<0.01
Surgery	F (1, 19) = 0.870; p = 0.363
Genotype x Surgery	F (1, 19) = 4.474; p<0.05
Grm5	
Genotype	F (1, 20) = 1.995; p = 0.173
Surgery	F (1, 20) = 0.533; p = 0.474
Genotype x Surgery	F (1, 20) = 0.501; p = 0.487
Homer1a	
Genotype	F (1, 19) = 7.742; p<0.05
Surgery	F (1, 19) = 0.041; p = 0.840
Genotype x Surgery	F (1, 19) = 4.930; p<0.05

RIGHT AMYGDALA

Ppara	
Genotype	F (1, 20) = 5.194; p<0.05
Surgery	F (1, 20) = 3.927; p = 0.061
Genotype x Surgery	F (1, 20) = 3.254; p = 0.086
Grm5	
Genotype	F (1, 20) = 5.900; p<0.05
Surgery	F (1, 20) = 10.349; p<0.01
Genotype x Surgery	F (1, 20) = 6.390; p<0.05

Kruskal-Wallis for RT-PCR data. Only when H was significant, Kruskal-Wallis was followed by U Mann Whitney with Bonferroni adjustment.

SPINAL CORD	
Ppara	H (3) = 5.507, p = 0.138
Rela	H (3) = 5.539, p = 0.136
Grm5	H (3) = 1.294, p = 0.731
Homer1a	H (3) = 3.497, p = 0.321
MPFC	
Grm5	H (3) = 14.125, p<0.01
RIGHT AMYGDALA	
Rela	H (3) = 0.087, p = 0.993
Homer1a	H (3) = 7.167, p = 0.067

Chapter #2

Unraveling the role of CB2 cannabinoid receptor presence to develop resilience or vulnerability to food addiction.

Ramírez-López Á, García-Blanco A, Navarrete F, García S, Manzanares J, Martín-García E, Maldonado R.

Unraveling the role of CB2 cannabinoid receptor presence to develop resilience or vulnerability to food addiction.

Under preparation

2.1. Abstract

Food addiction is characterized by the loss of behavioral control and compulsive food intake. Among other endogenous systems, the endocannabinoid system modulates cortical inputs implied in the inhibitory control over food intake. This study aimed to evaluate the cannabinoid receptor CB2 (CB2R) involvement in the reinforcing effects and food addiction-like behavior promoted by chocolate-flavored pellets. A widely validated operant training mouse model of food addiction was used in CD1 wild-type (WT) mice and mice lacking or overexpressing CB2R. Three hallmarks of addiction were evaluated at three different time points during the early, medium, and late training periods in this model: persistence of food-seeking during a period of non-availability, motivation for food, and compulsion in responding when the reward delivery was associated with a punishment. Each mouse was classified as resilient (0 criteria) or vulnerable (2-3 criteria) to this addictive-like behavior. Four additional phenotypic traits as factors of vulnerability to addiction were also evaluated during these three periods. The overexpression of CB2R, but not the deficiency, induced a vulnerable phenotype to food addiction after long-term exposure to highly palatable food. Furthermore, the lack of CB2R was a protective factor against the development of depressive-like behavior in addicted mice. Thus, CB2R seems involved in the predisposition to develop food addiction and may constitute a potential therapeutic target for compulsive food intake and the associated emotional affects of addictive behaviors.

2.2. Introduction

Randolph first described food addiction in 1956 (Randolph, 1956) and can be conceptualized as a compulsive eating behavior over palatable food. The prevalence of food addiction in the general population is between 2% and 12% among healthy body mass index individuals, but this prevalence increases among people suffering from obesity (18-24%), eating disorders (50%), or bulimia nervosa (85%) (Fernandez-Aranda *et al.*, 2018). As the consumption of highly caloric and palatable food is very present in our society, it has been a rising interest to study the neurobiological correlates and psychological effects of food addiction (Meule, 2015). Although food addiction is still not recognized in the last version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), new evidence have shown both neurobiological and behavioral equivalence with substance use disorders (Mancino *et al.*, 2015; Velázquez-Sánchez *et al.*, 2015; Volkow *et al.*, 2017; Domingo-Rodriguez *et al.*, 2020).

Substance use disorders are complex multifactorial diseases caused by a combination of genetic and environmental factors, including drug and natural rewards (Maldonado *et al.*, 2021a, 2021b). The rewarding effect produced by both drug and natural substances, such as palatable food, is a consequence of their action in the brain reward system by releasing dopamine in the nucleus accumbens (NAc) (Blum *et al.*, 2012; Volkow *et al.*, 2017). However, more than one brain area is implicated in the transition to addiction. In the case of food addiction, compulsive eating can be conceptualized as comprising three steps: (1) habitual overeating, (2) overeating to relieve a negative emotional state, and (3)

overeating despite adverse consequences (Moore *et al.*, 2017a, 2018). The basal ganglia, the extended amygdala, and the medial prefrontal cortex (mPFC) are the main areas that mediate these three stages of the addiction cycle, respectively (Koob and Volkow, 2010, 2016).

Mainly, glutamatergic frontostriatal projections rising from the mPFC to the NAc are responsible for the loss of inhibitory control in drug addiction and compulsive food intake (Chen *et al.*, 2013; Domingo-Rodriguez *et al.*, 2020). This glutamatergic activity is modulated by the endocannabinoid system since the absence of CB1R strengthens the release of glutamate in the NAc, leading to a resilience phenotype of food addiction (Domingo-Rodriguez *et al.*, 2020). Nevertheless, CB2R is also expressed in dopaminergic neurons (Liu *et al.*, 2017) and mediates central responses like depression, pain perception, and reward (Onaivi *et al.*, 2008; Shang and Tang, 2017). For this reason, CB2R modulation could have an important role in the habitual overeating and loss of inhibitory control stages of food addiction, as well as in the emergence of a negative affective state.

In this chapter, we aimed to elucidate the involvement of the CB2R in a food addiction model using two CB2R mutant mice lacking or overexpressing this cannabinoid receptor. We hypothesize that the lack of CB2R will lead to a protective resilient phenotype for food addiction, as previously reported in the case of CB1R absence (Domingo-Rodriguez *et al.*, 2020). Contrary, we expect that the overexpressing CB2R mice will result in a vulnerable phenotype with the emergence of a negative affect evidenced by emotional alterations.

2.3. Methods

Animals

CD1 wild-type (WT) male mice (n=42) were purchased from Charles River (France). Transgenic male mice overexpressing cannabinoid receptor 2 (CB2TG) (n=28) and cannabinoid receptor 2 knockout mice (CB2KO) (n=25) were kindly supplied by J. Manzanares laboratory (Instituto de Neurociencias, Universidad Miguel Hernández-CSIC Alicante, Spain). Briefly, male homozygote $CB2^{-/-}$ mice were initially generated on a C57BL/6J congenic background (provided by Nancy E. Buckley, Cal State Polytechnic University, Pomona, CA, USA), and the $CB2^{-/-}$ founders were crossed with outbred CD1 (Charles River, France) background (Buckley et al. 2000) for eight generations. Male mice overexpressing CB2R were on a CD1 congenic background. These mice were prepared as described in (Racz *et al.*, 2008a). All the behavioral experiments were conducted in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). At the beginning of the experiment, mice were 2 months old and weighed 40 ± 3 g. Mice were housed individually and maintained in a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%) environment. Food and water were available ad libitum, and mice were handled during the dark phase of a 12 h light/dark cycle (light on at 8:00 a.m., light off at 8:00 p.m.). All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive

(2010/63/EU). All the experiments were performed under blind and randomized conditions.

Operant behavior apparatus

Operant responding maintained by chocolate-flavored pellets was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA). The chambers were equipped with two retractable levers, one randomly assigned as the active lever and the other as the inactive for the entire experimental protocol. Pressing on the active lever resulted in a food pellet delivery paired with a stimulus-light (cue-light) located above the active lever, whereas pressing on the inactive lever had no consequences. A food dispenser equidistant between the two levers permitted the delivery of food pellets when pertinent. The chambers' floor consisted of metal bars able to conduct electrical discharges, serving as a contextual cue in the session of shock-associated cue the day after the shock session. During the rest of the self-administration sessions, a metal sheet with holes was placed above the grid floor. Thus, mice could discriminate between different contexts. The operant chambers were made of aluminum and acrylic and were housed inside soundproof boxes equipped with fans to provide ventilation and white noise.

Food pellets

During the operant conditioning sessions, animals received a 20 mg chocolate-flavored pellet after pressing the active lever, which is a highly palatable isocaloric pellet (TestDiet, Richmond, IN, USA). These pellets had a similar caloric value (3.44 kcal/g: 20.6% protein, 12.7% fat,

66.7% carbohydrate) to the standard maintenance diet provided to mice in their home cage (3.52 kcal/g: 17.5% protein, 7.5% fat, 75% carbohydrate) with some slight differences in their composition: chocolate flavor (2% pure unsweetened cocoa) and enhanced sucrose content (8.3% standard diet food vs. 50.1% highly palatable pellets). These pellets were presented only during the operant behavior sessions, and animals were maintained on standard chow for their daily food intake.

Experimental design

Self-administration session

A total of 95 mice were trained for 118 days in a self-administration behavior protocol for chocolate-flavored pellets. In the operant conditioning sessions, mice were under an FR1 schedule of reinforcement for 6 days (1 lever-press resulted in 1 pellet delivery) followed by 112 days of training on an increased FR to 5 (FR5) (5 lever-presses resulted in 1 pellet delivery) (**Fig. 1**). The beginning of each self-administration session was signaled by turning on a house light placed on the chamber's ceiling during the first 3 s. Daily self-administration sessions maintained by chocolate-flavored pellets lasted 1 h and were composed of 2 pellet periods (25 min each) separated by a pellet-free period (10 min). During the pellet periods, pellets were delivered contingently after an active response paired with a stimulus light (cue light). A time-out (TO) period of 10 s was established after each pellet delivery, where the cue light was off, and no reinforcer was provided after responding on the active lever. Responses on the active and inactive lever performed during the TO periods were recorded. In

contrast, the pellet-free period (PFP) was signaled by the illumination of the entire self-administration chamber, and no pellet was delivered after responding on any lever. Mice were returned to their home cages after each session.

As previously described (Martín-García et al., 2011), the operant responding was acquired when all the following conditions were achieved: (1) mice maintained a stable responding with less than 20% deviation from the mean of the total number of reinforcers earned in 3 consecutive sessions (80% of stability); (2) at least 70% responding on the active lever; and (3) a minimum of 10 reinforcers per session.

Three addiction criteria

The food addiction criteria were evaluated at three different time points as previously described (Mancino *et al.*, 2015): early (5-22 FR5 sessions), medium (48-62 FR5 sessions), and late (98-112 FR5 sessions) periods (**Fig. 1**). The food addiction criteria gathered the main hallmarks of addiction based on DSM-IV (Deroche-Gamonet *et al.*, 2004), specified in DSM-5 and now included in the food addiction diagnosis through the YFAS 2.0 (Gearhardt *et al.*, 2016):

Persistence to response: persistent desire or unsuccessful efforts to cut down displayed by continuous food-seeking behavior even if the food reward is signaled as not available. It is measured by the number of non-reinforced active responses during the PFP (10 min) on the 3 consecutive days before the progressive ratio (PR).

Motivation: considerable effort and time spent in obtaining the reward. It is measured by the PR schedule of reinforcement. The

response required to earn one single pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000, 1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, and 5500. The maximal number of responses that the animal performs to obtain one pellet was the last event completed, referred to as the breaking point. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h.

Compulsivity: continued use despite adverse consequences. It is the resistance to punishment when chocolate-flavored pellets intake is maintained despite its negative consequences. Mice were placed in a self-administration chamber without the metal sheet with holes and consequently with the grid floor exposed (contextual cue). During this session, mice underwent an FR5 schedule in which they received an electric foot-shock (0.18 mA, 2 s) after 4 responses and received an electric foot-shock (0.18 mA, 2 s) and a pellet paired with the cue light after the 5th response. The schedule was reinitiated after 10 s pellet delivery (time-out period) and after the fourth response if mice did not perform the fifth response within 60 s. The total number of shocks performed in 50 min was used to evaluate compulsivity-like behavior, previously described as resistance to punishment (Deroche-Gamonet et al., 2004; Mancino et al., 2015).

Establishment of mice subpopulations

After performing the three behavioral tests to measure the food addiction behavior, mice were categorized as food addicted or non-addicted depending on the number of positive criteria they achieved.

Results were expressed as individual values with the median and the interquartile range. A mouse was considered positive for a particular addiction criterion when the score of the specific behavioral test was above the 75th percentile of the normal distribution of the WT control group. Mice that achieved 2 or 3 addiction criteria were considered addicted animals, and mice that achieved 0 or 1 addiction criteria were considered non-addicted animals, as previously reported (Mancino *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020).

Behavioral tests to evaluate addiction-like phenotypic traits

After the categorization in food addicted and non-addicted mice, 4 additional phenotypic traits as factors of vulnerability to addiction were also evaluated in each period (early, medium, late):

(1) ***Impulsivity***

The inability to stop a response once it is initiated was measured as the number of non-reinforced active responses during the TO period (10 s) after each pellet delivery. This impulsivity-like behavior was delimited to the 3 consecutive days before the progressive ratio.

(2) ***Cognitive flexibility***

Cognitive flexibility tested the ability to modify the operant behavior when the active and the inactive levers were reversed in a single training session without previous learning. It was measured by the number of active-reversed responses (previous inactive lever in a normal training session) performed in 1 h.

(3) ***Appetitive associative learning***

The cue-induced food-seeking test, which consisted of a 90 min session, assessed the conditioning to an appetitive stimulus (cue-light). In the first 60 min, all active and inactive lever-presses were recorded but produced no consequences. In the next 30 min, the cue-light associated with pellet delivery during a normal self-administration session was illuminated but with no contingent pellet reinforcement. To signal the change in the schedule, the cue light was presented twice non-contingently and for 4 s.

(4) Aversive associative learning

To study the conditioning to an aversive stimulus (grid floor), non-reinforced active responses during the following session after the shock-test were measured. Mice were placed in the self-administration chamber for 50 min with the same grid floor used during the shock test. However, during this session, pressing the active lever had no consequences: no shock, no chocolate-flavored pellets, and no cue-light.

Locomotor activity

Locomotor activity was evaluated using individual locomotor activity boxes 10.8 cm width × 20.3 cm length × 18.6 cm high equipped with infrared sensors to detect horizontal locomotor activity and an infrared plane to detect rearings (Imetronic, Pessac, France). The boxes were provided with a removable cage, a sliding floor, a trough, and a bottle at the front. Mice were placed in the boxes for 1 h, and the kinetics of the total activity (number of beam breaks) was recorded in blocks of 10

min. A locomotor activity study was performed with no previous habituation to the activity boxes.

Anxiety-like behavior

The elevated plus-maze test was used to evaluate anxiety-like behavior at the end of the long-term operant training maintained by chocolate-flavor pellets. A black Plexiglas apparatus consisting of 4 arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in a cross from a neutral central square (5 x 5 cm) elevated 40 cm above the floor was used. Light intensity in the open and closed arms was 45 and 5 lux, respectively. Mice were placed in the central square facing one of the closed arms and tested for 5 min. The time spent in the open and closed arms of the maze was determined as a measure of anxiety-like behavior, whereas the total entries in the open and closed arms were considered a measure of locomotor activity, as previously reported (La Porta *et al.*, 2015).

Depressive-like behavior

Depressive-like behavior was evaluated at the end of the long-term operant training maintained by chocolate-flavor pellets using the forced swimming test (Porsolt and Bertin, 1977). Briefly, mice were individually placed into a glass cylinder (17.5 x 12.5 cm) filled 15 cm high with water (22 ± 1°C). Mice were subjected to forced swimming for 6 min, and the total duration of immobility, disregarding small hind limb movements to keep the head above water, was measured during the last 4 min when mice show a sufficiently stable level of immobility.

Statistics

Principal component analysis

The principal component analysis (PCA) technique was used to evaluate the multidimensional data obtained in mice chronically trained with chocolate-flavored pellets. PCA and varimax rotation were conducted using the 3 addiction-like criteria and the 4 phenotypic traits considered as factors of vulnerability to addiction and were dimensionality reduced to the minimum number of components that best explain and maximizes the variance present in the data set. An eigenvalue greater than 1 was set as selecting components criterion.

Statistical analysis of behavioral data

IBM SPSS 19 (SPSS Inc., Chicago, USA) was used to analyze all the data. ANOVA with repeated measures was used when required to test the evolution over time with the subsequent post hoc analysis with genotype (Bonferroni). For multiple group comparisons, one-way ANOVA or U Mann-Whitney were used depending on the distribution defined by the Shapiro-Wilk normality test. When two groups were compared, Student T-test or U Mann-Whitney were used depending on the distribution defined by the Shapiro-Wilk normality test. Bonferroni *post hoc* analysis was performed when pertinent. Chi-square analyses were performed to compare the percentage of addicted mice with the non-addicted ones, comparing the observed frequencies with the frequencies obtained in the control WT group. The Pearson correlation coefficient was used to analyze the relationship between the 3

addiction-like criteria and the final addiction criteria. A probability of 0.05 or less was considered statistically significant.

2.4. Results

Experimental protocol

WT (n = 42), CB2KO (n = 25), and CB2TG (n = 28) mice were trained in the operant chambers under an FR1 schedule of reinforcement during 6 sessions followed by 112 sessions under FR5 to acquire an operant responding maintained by chocolate flavored-pellets (**Fig. 1**).

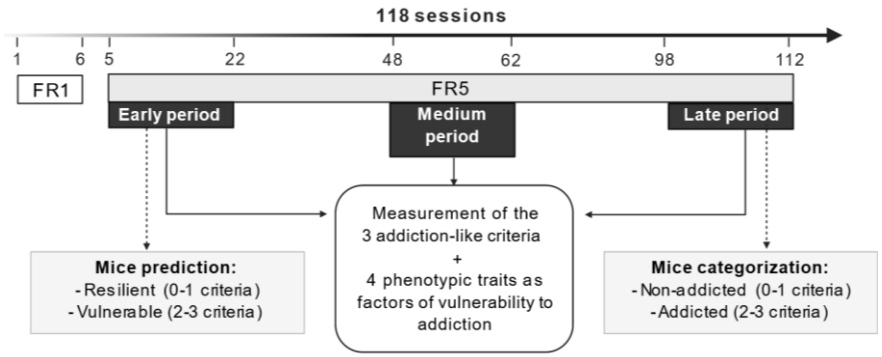


Figure 1. Experimental timeline of the food addiction mouse model. Mice were trained to obtain chocolate-flavored pellets under a fixed ratio (FR) 1 schedule of reinforcement on 1 h daily self-administration sessions for 6 days followed by 112 days on an FR5 (a total of 118 training sessions). In the FR5 training period, 3-time points were considered, early (5-22 training days), medium (48-62 training days), and late (98-112 training days), to measure the addiction criteria (persistence to response, motivation, and compulsivity) and classified mice as non-addicted (0-1 criteria) and addicted (2-3 criteria). Four phenotypic traits were measured in these 3-time points as additional factors of vulnerability to food addiction (impulsivity, cognitive flexibility, and aversive and appetitive associative learning). Adapted from (Domingo-Rodriguez *et al.*, 2020; Maldonado *et al.*, 2021b).

Evaluation of body weight evolution and locomotor activity

At the beginning of the experiment (week 0), all animals weighed 40 ± 3 g. However, significant differences in body weight were reported during the entire experiment, with CB2TG mice showing lower body weight than WT and CB2KO mice (repeated measures ANOVA, genotype effect, $p < 0.001$; Bonferroni, $p < 0.001$, **Fig. 2A**). In contrast, WT and CB2KO mice gained progressive weight over time without significant differences among them (repeated measures ANOVA, genotype effect, $p < 0.001$; Bonferroni, n. s., **Fig. 2A**).

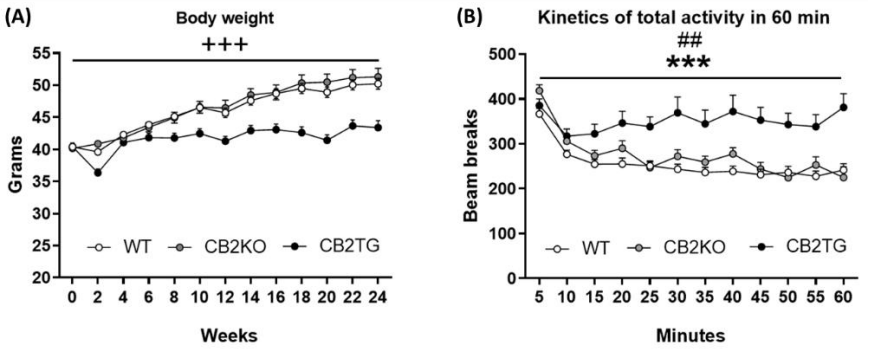


Figure 2. Additional variables to measure. (A) Body weight. Weekly measurements of body weight in grams. CB2TG mice showed a reduced body weight compared to WT and CB2KO mice. **(B)** Kinetics of total activity. Horizontal locomotor activity measured by beam breaks represented in 10-min blocks during 1 h. CB2TG mice showed hyperlocomotion compared to WT and CB2KO mice. Data are expressed as mean ± SEM (WT n = 42, CB2KO n = 25, CB2TG n = 28). +++p<0.001 vs. CB2TG, ***p<0.001 vs. WT, ##p<0.001 vs. CB2KO (Repeated measure ANOVA, Bonferroni).

Comparisons in locomotor activity between genotypes were also evaluated in the early period during 1h, the same time that lasts a daily training session. CB2TG mice revealed a higher horizontal locomotor

activity in comparison to WT and CB2KO mice (repeated measures ANOVA, genotype effect, $p < 0.001$; Bonferroni, $p < 0.001$ vs. WT, $p < 0.01$ vs. CB2KO, **Fig. 2B**), indicated by the number of total beam breaks. In contrast, CB2KO mice showed similar kinetics of horizontal activity compared with WT animals (repeated measures ANOVA, genotype effect, $p < 0.001$; Bonferroni, n. s., **Fig. 2B**).

Acquisition of operant training maintained by chocolate flavored-pellets

During FR1, all groups increased the number of reinforcers across sessions without significant differences between genotypes (repeated measures ANOVA, genotype effect, n. s., **Fig. 3**). Next, when the effort to get one single pellet was increased to FR5, all genotypes showed a progressive increase of the number of reinforcers across time. Interestingly, the number of reinforcers was significantly reduced in CB2KO compared to WT mice over the whole FR5 period (repeated measures ANOVA, genotype effect, $p < 0.05$; Bonferroni, $p < 0.05$, **Fig. 3**). In contrast, CB2TG mice showed a reduced pellet consumption compared to WT mice only in the early period (U Mann-Whitney, $p < 0.05$, **Fig. 4**), losing this significant difference from the medium period onwards (ANOVA, n. s., **Fig. 4**). Furthermore, in the late period, CB2TG mice also showed a significantly higher number of reinforcers compared to CB2KO mice (repeated measures ANOVA, genotype effect, $p < 0.05$; Bonferroni, $p < 0.05$, **Fig. 3**). All genotypes significantly increased the amount of pellets intake during the medium and late periods compared to the early period (Wilcoxon, $p < 0.001$, **Fig. 4**). Moreover, CB2R mutants also increased pellet consumption from the

medium to the late period, especially in CB2TG mice (Paired t-test, $p < 0.001$, **Fig. 4**). These results suggest that chocolate-flavored pellets were less reinforcing for CB2KO mice since the early period suggesting that the loss of CB2R may be a predisposing protective factor. In contrast, the overexpression of CB2R has a dual effect with protection in the early period and may represent a risk factor in the late period after long-term exposure to palatable food.

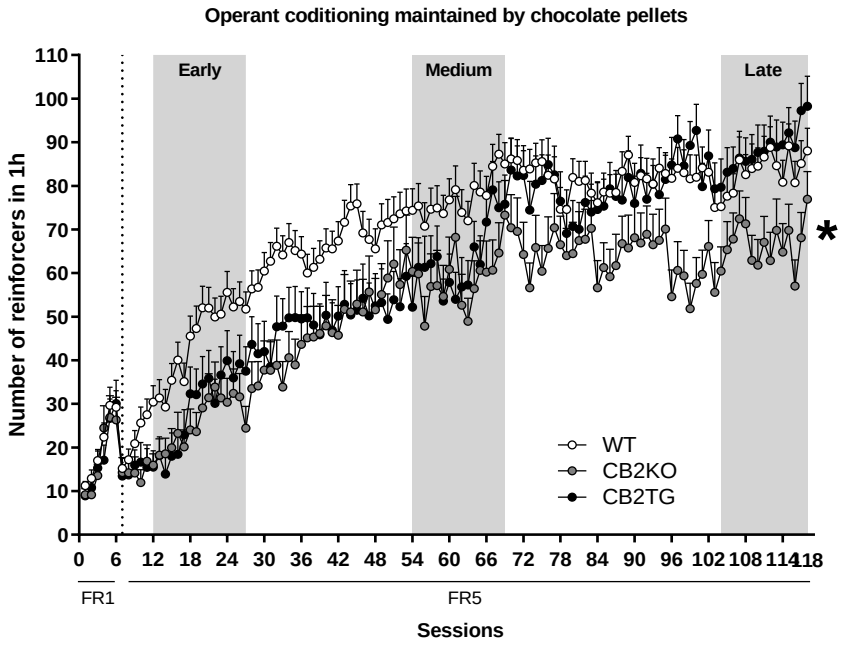


Figure 3. The number of chocolate-flavor pellets obtained in each daily self-administration session. CB2TG mice showed a reduced number of reinforcers during 1 h of operant training sessions maintained by chocolate-flavored pellets compared to WT mice only in the early period, whereas CB2KO mice exhibited this difference over the entire FR5 period. Data are expressed as mean ± SEM (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ vs. WT (Repeated measures ANOVA, Bonferroni test).

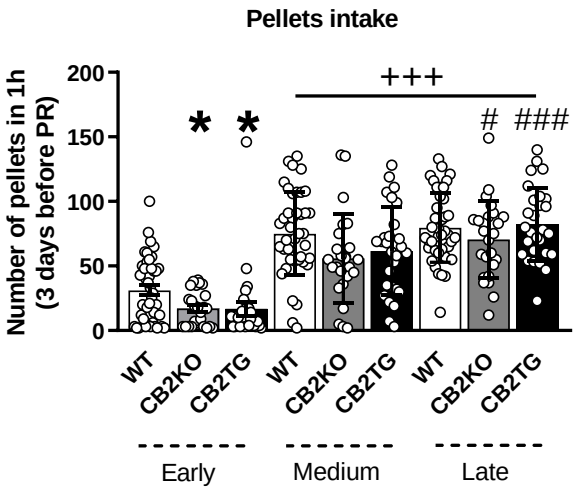


Figure 4. Number of chocolate-flavored pellets intake during the 3 consecutive training sessions before the progressive ratio test. All genotypes increased the amount of pellets intake during the medium and late periods compared to the early period. Deeply, CB2R mutants progressively obtained more pellets in the late than in the medium period and showed a decreased pellet consumption compared to WT mice only in the early period. Data are expressed as mean ± SEM (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ vs. WT (Kruskal-Wallis, U Mann Whitney), +++ $p < 0.001$ vs. early period (Wilcoxon), ## $p < 0.05$, ### $p < 0.001$ vs. medium period (Paired t-test).

Most of the animals achieved the acquisition criteria during the FR5, WT (91.30%), CB2KO (96.5%), and CB2TG (90.32%), after an average of 17.36 ± 2.56, 26.72 ± 4.87, and 29.68 ± 5.40 sessions, respectively (**Fig. 5**). No significant differences in the day of acquisition between genotypes indicated similar acquisition levels of the operant conditioning learning driven by chocolate-flavored pellets (Kruskal-Wallis n. s., **Fig. 5**).

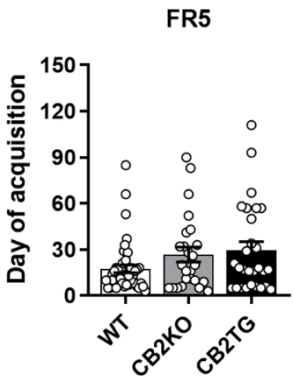


Figure 5. Day of acquisition of the operant conditioning maintained by chocolate-flavor pellets on the FR5 period. Data are expressed as mean ± SEM (WT n = 42, CB2KO n = 25, CB2TG n = 28). (Kruskal-Wallis).

Differences between genotypes in the three addiction criteria

All groups of animals were tested for the 3 behaviors used to evaluate the addiction criteria during the early (5-22 sessions), medium (48-62 sessions), and late (98-112 sessions) periods of the operant training (**Fig. 1**).

In the **early period**, significant genotype differences were found in the criterion of persistence to response, evaluated by the number of non-reinforced active responses during the PFP, and the motivation test, defined by the breaking point obtained during the PR schedule. Both CB2KO and CB2TG mice showed a reduced motivation compared to WT mice (U Mann-Whitney, $p < 0.05$, **Fig. 6B**), whereas only CB2TG mice showed significantly less persistence to response in seeking palatable food compared to WT mice (U Mann-Whitney, $p < 0.01$ **Fig. 6A**). Non-significant genotype differences were observed in the compulsivity test, which was evaluated by the number of active responses associated with a foot-shock delivery (Kruskal-Wallis, n. s., **Fig. 6C**). In the **medium period**, both CB2KO and CB2TG mice maintained a reduced motivation in comparison to WT mice (U Mann-Whitney, p <

0.05, **Fig. 6E**). Moreover, CB2TG mice showed a higher compulsivity for chocolate-flavored pellets than WT and CB2KO mice (U Mann-Whitney, $p < 0.01$, **Fig. 6F**). However, non-significant genotype differences were observed in the persistence to response test during the medium period (Kruskal-Wallis, n. s., **Fig. 6A**). Finally, in the **late period**, CB2TG mice showed significantly more persistence to response than WT mice (U Mann-Whitney, $p < 0.05$, **Fig. 6G**). No other significant differences between genotypes were observed in the motivation and compulsivity tests during this period (Kruskal-Wallis, n. s., **Fig. 6H-I**).

Using the results of three food addiction-like criteria in the early, medium, and late periods, mice were individually categorized as non-addicted (covering 0-1 criteria) or addicted (covering 2-3 criteria) as previously reported (Mancino *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020). A mouse was considered positive for an addiction-like criterion when its score for each behavior was equal to or beyond the 75th percentile of the distribution of the WT group (colored circles, **Fig. 6**).

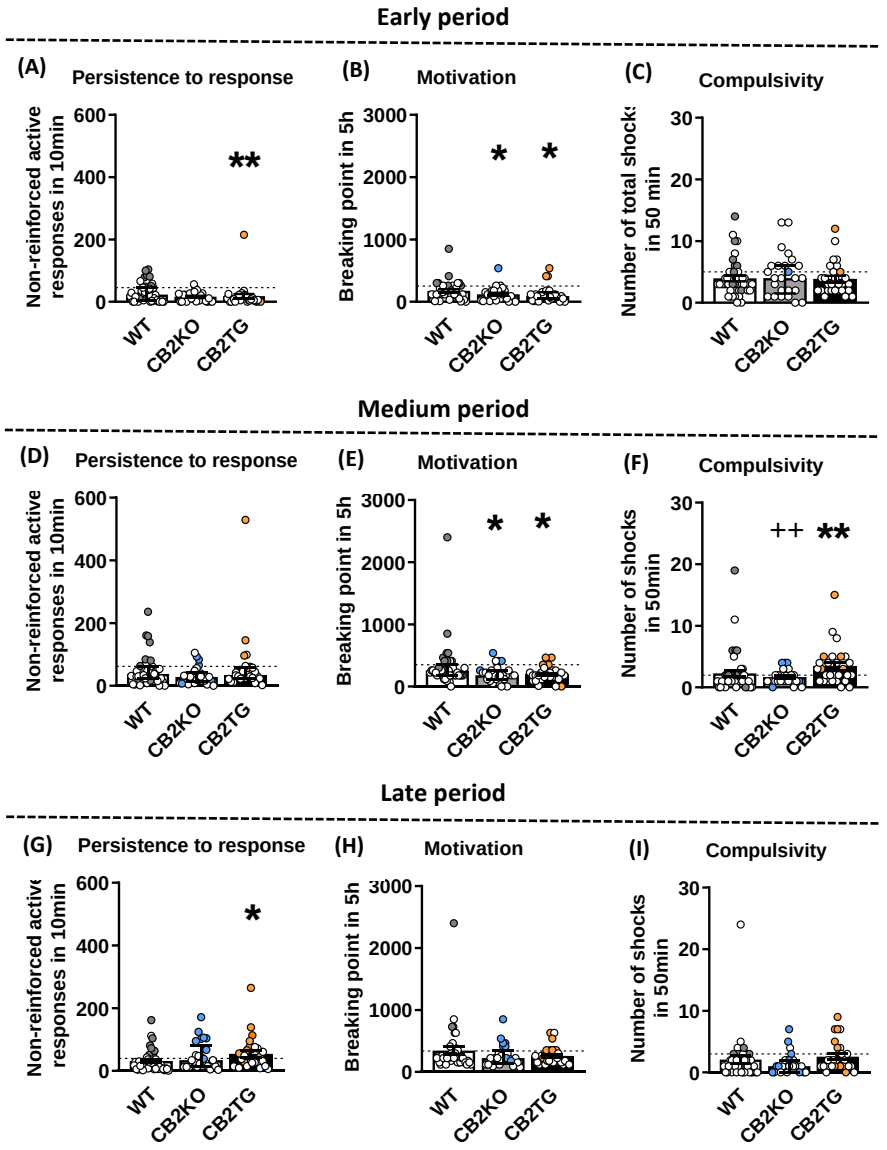
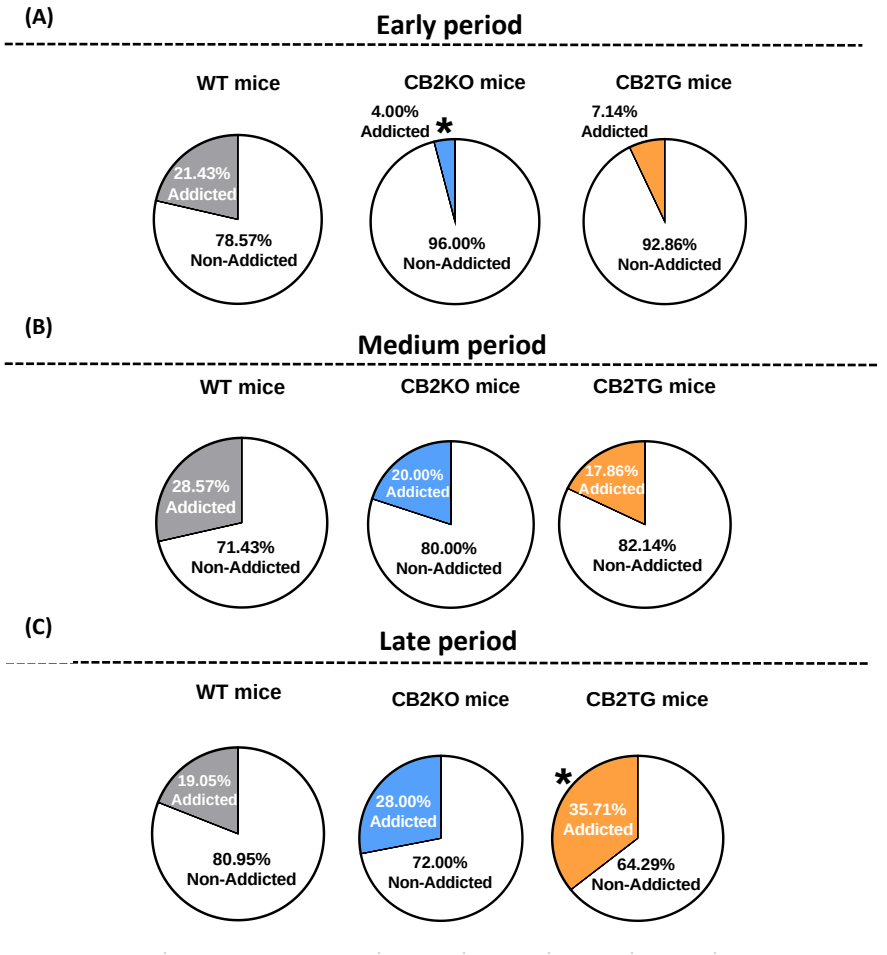


Figure 6. Behavioral tests for the three addiction criteria in the early, medium, and late periods. (A, D, G) Persistence to response. **(B, E, H)** Motivation. **(C, F, I)** Compulsivity. Dashed lines indicated the 75th percentile of the distribution of the WT group which is used as a threshold to consider a mouse positive for one criterion (colored circles). Data are expressed as individual values with median ± interquartile range (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ vs. WT, ** $p < 0.01$ vs. WT, ++ $p < 0.01$ vs. CB2TG (Kruskal-Wallis, U Mann Whitney).

During the **early training**, only 4.00% and 7.14% of the mutant mice, CB2KO and CB2TG, respectively, achieved 2-3 criteria compared with the 21.43% addicted WT mice. However, only CB2KO mice exhibited a resilient phenotype to food addiction compared to the WT control group (Chi-square, $p < 0.05$, **Fig**. **7A**). During the **medium training**, the percentage of addicted mice increased in all genotypes (WT = 28.57%, CB2KO = 20.00%, CB2TG = 17.86% covering 2-3 criteria), especially for CB2KO mice that lost the protection to food addiction-like behavior (**Fig**. **7B**). Chi-square tests did not reveal significant differences in the percentage of mice subpopulations between genotypes during the medium period. Finally, during the **late training**, 28.00% of CB2KO mice reached 2-3 criteria (addicted mice) without significant differences with the 19.05% of WT addicted mice (Chi-square, n. s., **Fig**. **7C**). However, the long-term exposure to highly palatable pellets promotes a strong operant seeking behavior in CB2TG mice, since 35.71% of CB2TG animals showed a vulnerable phenotype to food addiction compared to WT mice (Chi-square, $p < 0.05$, **Fig. 7C**). Thus, the overexpression of CB2R represents a risk factor to food addiction-like behavior after long-term exposure to palatable food.



	Non-addicted		Addicted	
	0 crit	1 crit	2 crit	3 crit
WT	50.0%	30.95%	14.29%	4.76%
CB2KO	52.0%	20.0%	24.0%	4.0%
CB2TG	28.57%	35.71%	28.57%	7.14%

Figure 7. Percentage of addicted and non-addicted mice classified based on food addiction-like criteria scoring. (A) Early period **(B)** Medium period **(C)** Late period. The table summarizes the final distribution of the different criteria subgroups in percentages based on the performance at the late period. (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ (Chi-square).

Once animals were categorized as addicted and non-addicted, their behavioral evolution for the three-addiction criteria test was separately analyzed in each genotype. WT mice classified as addicted showed higher values than non-addicted mice in each addiction criteria test from the early to the late period in the case of persistence to response (U Mann-Whitney, $p < 0.001$, **Fig. 8A**) and compulsivity (U Mann-Whitney, $p < 0.05$, **Fig. 8A**), and in the early and medium periods for motivation (U Mann-Whitney, $p < 0.001$, **Fig. 8A**). In contrast, CB2KO mice exhibited significant differences between addicted and non-addicted mice from the medium period in the motivation test (T-Test, $p < 0.01$, $p < 0.001$, **Fig. 8B**) and only in the late period in the persistence to response (T-Test, $p < 0.001$, **Fig. 8B**). No significant differences were found in the compulsivity test between addicted and non-addicted CB2KO mice (U Mann-Whitney, n. s., **Fig. 8B**). In CB2TG mice, remarkable differences between addicted and non-addicted animals were observed in motivation during the entire experimental protocol (U Mann-Whitney, $p < 0.01$, $p < 0.001$, **Fig. 8C**), in the persistence to response of the medium and late periods (U Mann-Whitney, $p < 0.01$, $p < 0.001$, **Fig. 8C**) and only in the medium period of the compulsivity test (U Mann-Whitney, $p < 0.05$, **Fig. 8C**). These results demonstrated that addicted and non-addicted mice performed differently in the three-addiction criteria test, especially WT and CB2TG mice, which strengthened the categorization.

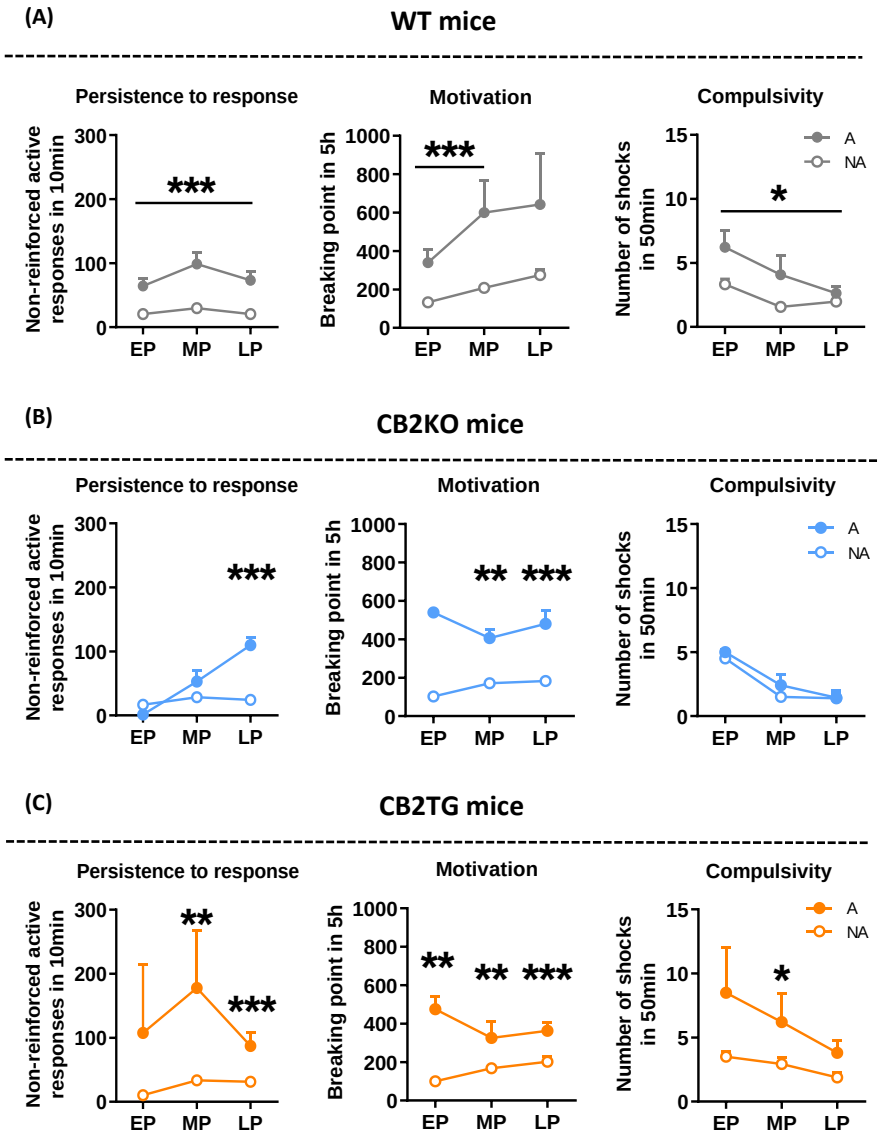


Figure 8. Behavioral evolution of classified addicted and non-addicted mice in the addiction criteria tests over the early, medium, and late periods. (A) WT mice **(B)** CB2KO mice **(C)** CB2TG mice. Addicted mice (WT n = 8, CB2KO n = 7, CB2TG n = 10). Non-addicted (WT n = 34, CB2KO n = 18, CB2TG n = 18). Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (T-Test or U Mann-Whitney when pertinent). (A: addicted, NA: non-addicted, EP: Early period, MP: Medial period, LP: Late period).

The number of addicted mice that coincided between periods was calculated to evaluate the predictive value of mice categorization for the early and medium periods. The percentage of WT mice finally classified as addicted in the late period matched with that obtained in both early and medium periods (50%) (**Fig. 9**). However, the degree of coincidence between the medium and late periods of CB2KO (14.28%) and CB2TG (30.0%) addicted mice was higher than the comparison between the early and late periods (0.0% and 10.0%, respectively) (**Fig. 9**). Therefore, the medium period displayed a more predictable value to categorize addicted mice than the early period.

Degree of coincidence: Addicted mice

	E → M	E → L	M → L
WT	41.6%	50.0%	50.0%
CB2KO	20.0%	0.0%	14.28%
CB2TG	40.0%	10.0%	30.0%

Figure 9. Percentages of addicted mice that coincide between periods. (E: Early period, M: Medium period, L: Late period).

Furthermore, positive correlations were observed between the performance in each addiction-like behavioral test and the final addiction criteria achieved during the late training (**Fig. 9A-C**). Interestingly, strong associations were found between CB2TG classified addicted mice, but no CB2KO and WT mice, and the number of total shocks received in 50 min (**Fig. 9C**), suggesting a compulsive food intake behavior of CB2TG addicted mice despite negative consequences.

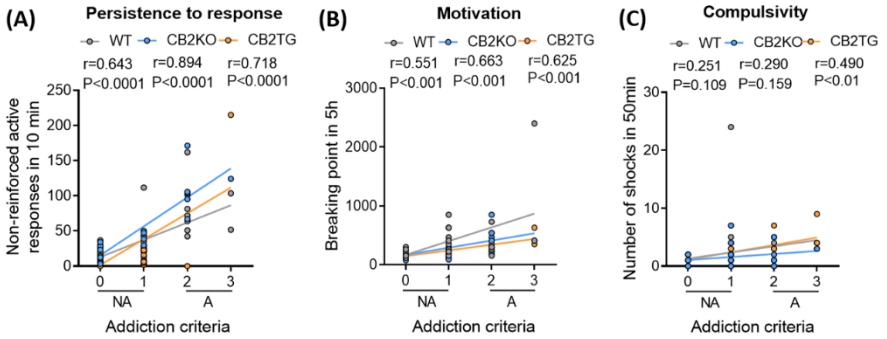


Figure 9. Correlations. Pearson correlations between individual addiction-like criteria and **(A)** Non-reinforced active responses in 10 min, **(B)** Breaking point in 5 h, **(C)** Number of shocks in 50 min in the late period. (WT n = 42, CB2KO n = 25, CB2TG n = 28). (NA: non-addicted, A: addicted).

Differences between genotypes in the addiction-like phenotypic traits

Four additional phenotypic traits as vulnerability factors to addiction were also evaluated to study the addictive phenotype more deeply:

1. *Impulsivity* was measured by the number of non-reinforced active responses performed during the TO period (10 s) after each pellet delivery.
2. *Cognitive flexibility* was evaluated by the number of active-reversed responses during a normal self-administration session where the active and the inactive levers were reversed, referred to as reversal test (1 h).
3. *Aversive associative learning* tested the influence of the shock-associated cue (the grid floor) to suppress pellets seeking the day after the compulsivity test, referred to as the post-shock test (1 h).

4. *Appetitive associative learning* evaluated the association between the cue-light present during pellet delivery (conditioned stimulus) and food, referred to as cue-induced food-seeking test (90 min).

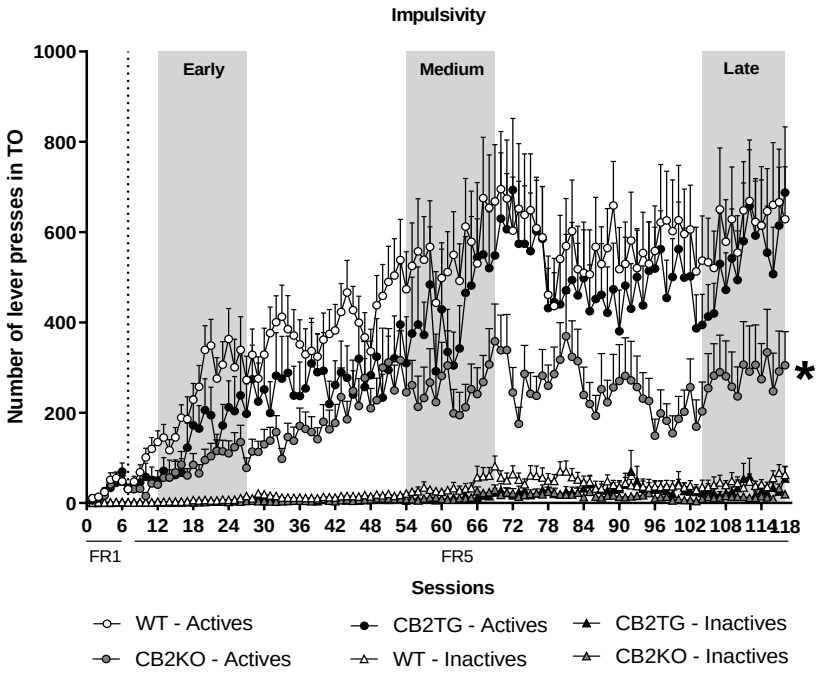


Figure 10. Number of active and inactive lever presses during training sessions. CB2KO mice showed a reduced number of non-reinforced active responses during 1 h of operant training sessions compared to WT mice, whereas CB2TG and WT mice exhibited similar responses. All genotypes showed good discrimination between active and inactive levers. Data are expressed as mean ± SEM (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ vs. WT (Repeated measures ANOVA, Bonferroni test).

As a measure of impulsivity-like behavior, active and inactive lever presses during the 10 s of the TO period were also recorded over each self-administration session. CB2KO mice showed a reduced number of non-reinforced active responses compared to WT mice among all FR5

sessions (repeated measures ANOVA, genotype effect, $p < 0.05$; Bonferroni, $p < 0.05$, **Fig. 10**), suggesting that CB2KO mice are less impulsive than control WT animals. In contrast, CB2TG mice exhibited a similar number of active lever presses in the TO period compared to WT mice (repeated measures ANOVA, genotype effect, $p < 0.05$; Bonferroni, n. s., **Fig. 10**). All genotypes showed good discrimination between the active and the inactive levers (**Fig. 10**).

During the **early period**, only CB2KO mice showed significantly less impulsivity and less sensitivity to an aversive and an appetite conditioned stimulus compared to WT mice (U Mann-Whitney, $p < 0.01$, **Fig. 11A**). CB2TG mice exhibited similar behavioral responses in the mentioned test compared to WT animals (Kruskal-Wallis, n. s., **Fig. 11A**), except for the appetitive conditioning learning (U Mann-Whitney, $p < 0.05$, **Fig. 11A**). Regarding the reversal test, non-significant genotype differences were observed in cognitive flexibility (Kruskal-Wallis, n. s., **Fig. 11A**). During the **medium period**, CB2KO mice still presented decreased impulsivity-like behavior when compared to WT mice (U Mann-Whitney, $p < 0.01$ vs. WT, $p < 0.05$ vs. CB2TG, **Fig. 11B**), but no significant differences were observed in other addiction-like phenotypic traits within CB2KO or CB2TG mice in comparison to WT mice (Kruskal-Wallis, n. s., **Fig. 11B**). Finally, in the **late period**, significant genotype differences were only observed in the impulsivity-like behavior where CB2KO mice showed significantly less impulsivity than WT and CB2TG mice (U Mann-Whitney, $p < 0.05$, **Fig. 11C**). Non-significant genotype differences were found in any other phenotypic traits test Kruskal-Wallis, n. s., **Fig. 11C**).

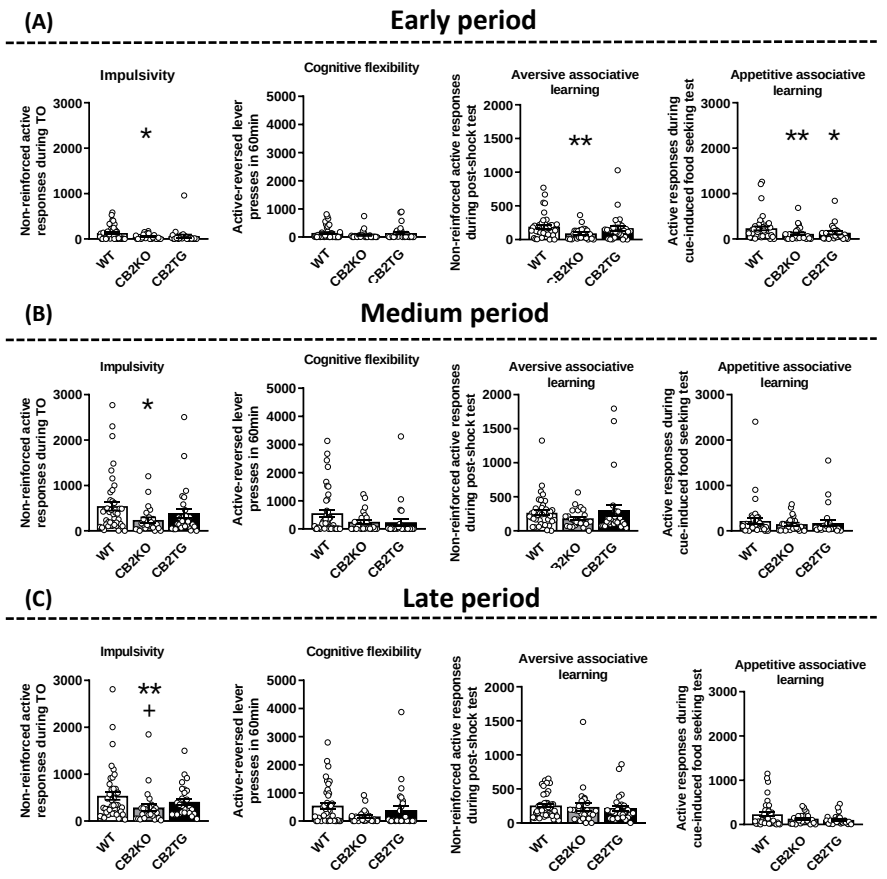


Figure 11. Analysis of the four addiction-like phenotypic traits in the early (A), medium (B) and late (C) periods. Impulsivity, cognitive flexibility, aversive associative learning, and appetitive associate learning. (WT n = 42, CB2KO n = 25, CB2TG n = 28). * $p < 0.05$ vs. WT, ** $p < 0.01$ vs. WT, + $p < 0.05$ vs. CB2TG (Kruskal-Wallis, U Mann Whitney).

Principal component analysis revealed differential patterns of behavioral factor loadings in food addiction-like behavior in mice

Evaluation of the links between the different behavioral addiction-like criteria and phenotypic traits was performed in each genotype using principal component analysis (PCA). In the **WT group**, the percentage of variance explained by the two principal components (PC) was

44.83% (PC1) and 15.21% (PC2). The main addiction criterion loading in PC1 for WT mice was motivation, whereas compulsivity was the predominant criterion in PC2. Among the four phenotypic traits, impulsivity and both appetitive and aversive associative learnings in WT mice had the highest loading in PC1, whereas cognitive flexibility showed the lowest loading in PC1 (**Fig. 12A-E**). For the **CB2KO group**, PC1 explained 47.99% of the variance and PC2 the 20.48%. Like WT mice, the main addiction criterion loading in PC1 of CB2KO mice was motivation, followed by the persistence to response. In contrast, the compulsivity criterion did not meet the proposed loading criterion (0.40) in CB2R mutants (Field, 2018). Regarding the phenotypic traits, impulsivity and appetitive associative learning in CB2KO mice weighted more in PC1, whereas cognitive flexibility weighted more in PC2 (**Fig. 13A-E**). Lastly, the percentage of variance explained by PC1 was 40.96% and 17.77% by PC2 in the **CB2TG group**. Unlike WT mice, the main addiction criterion loading in PC1 for CB2R transgenic mice was the persistence to response, followed by motivation, and the compulsivity criterion showed the lowest loading in PC1. Among the four phenotypic traits, aversive associative learning and impulsivity in CB2TG mice had the highest loading in PC1, whereas appetitive associative learning showed the main loading in PC2. The cognitive flexibility of CB2TG mice did not meet the proposed loading criterion (0.40) (Field, 2018) (**Fig. 14A-E**).

WT mice

(A)

Variables	Factor loadings: Principal component 1 (44.84 %)	Factor loadings: Principal component 2 (15.21%)
Persistence to response	0.57	-0.42
Motivation	0.89	0.03
Compulsivity	0.09	0.91
Impulsivity	0.84	-0.01
Cognitive flexibility (errors)	0.40	0.00
Appetitive associative learning	0.77	0.21
Aversive associative learning (errors)	0.75	-0.15

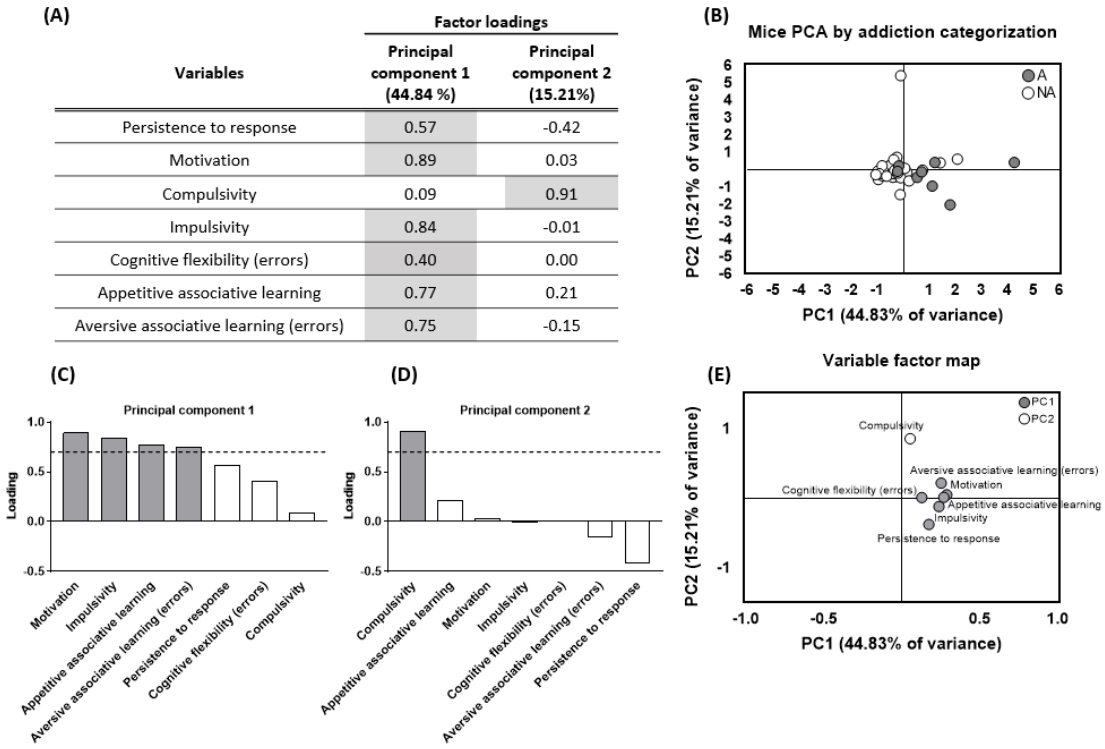


Figure 12. Principal components analysis (PCA) of food addiction phenotype in WT mice. (A) PCA of the three addiction criteria and the four phenotypic traits with factor loadings of principal component (PC) 1 (44.83%) and PC2 (15.21%). Concerning the addiction criteria, a dissociation between persistence and motivation for one side and compulsivity for the other was observed. The four phenotypic traits weighted more in PC1. **(B)** Mice subjects clustered by presence addicted or non-addicted on the space yielded two components of the PCA that account for the maximum data variance. (n=8 addicted (A) mice, n=34 non-addicted (NA) mice). **(C-D)** Graphs with the order of factor loading of the different variables in the PC1 **(C)** and PC2 **(D)**. The dashed horizontal line marked loadings > 0.7 mainly contributing to the component. **(E)** Behavioral tests clustered according to the loading in two components of the PCA.

(A)

Variables	Factor loadings: Principal component 1 (47.99%)	Principal component 2 (20.48%)
Persistence to response	0.82	-0.04
Motivation	0.89	0.34
Compulsivity	-0.12	0.30
Impulsivity	0.86	-0.09
Cognitive flexibility (errors)	0.13	0.89
Appetitive associative learning	0.81	-0.20
Aversive associative learning (errors)	0.69	0.61

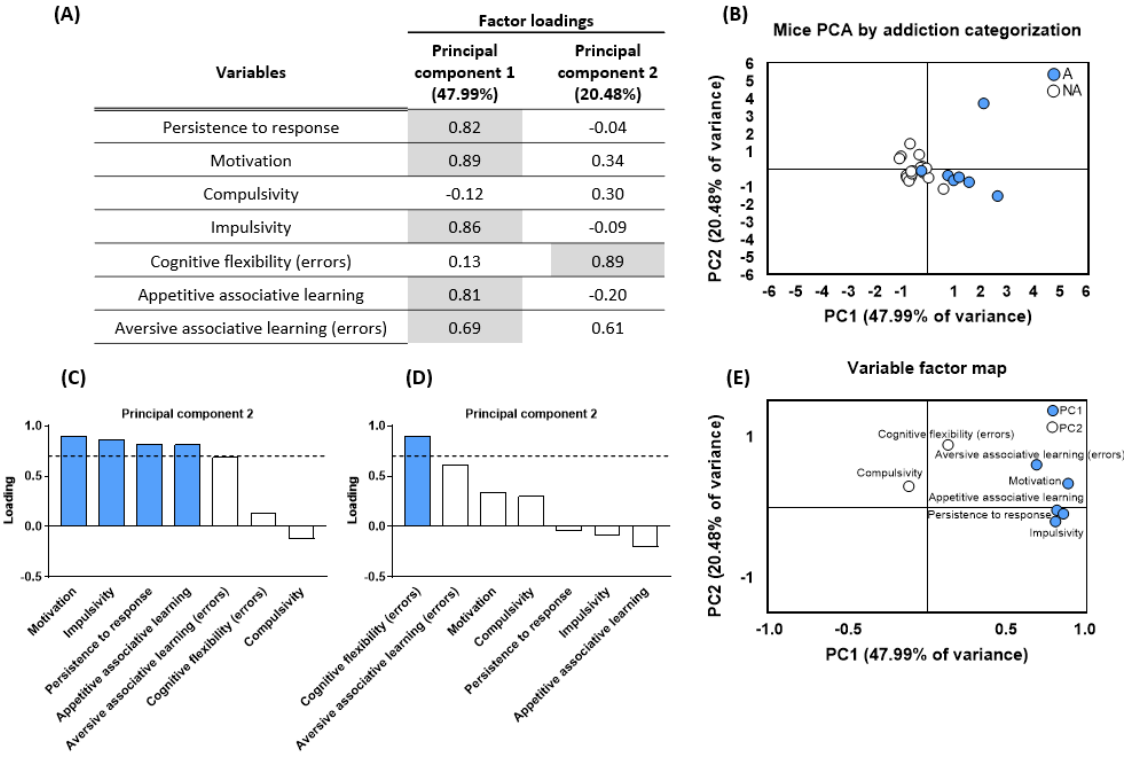


Figure 13. Principal components analysis (PCA) of food addiction phenotype in CB2KO mice. (A) PCA of the three addiction criteria and the four phenotypic traits with factor loadings of principal component (PC) 1 (47.99%) and PC2 (20.48%). Concerning the addiction criteria, a dissociation between persistence and motivation for one side and compulsivity for the other was observed. However, the compulsivity criterion did not meet the proposed loading criterion (0.40) (Field, 2018). The phenotypic traits weighted more in PC1, except for the cognitive flexibility that weighted more in PC2. **(B)** Mice subjects clustered by presence addicted or non-addicted on the space yielded by two components of the PCA that account for the maximum data variance. (n=7 addicted (A) mice, n=18 non-addicted (NA) mice). **(C-D)** Graphs with the order of factor loading of the different variables in the PC1 **(C)** and PC2 **(D)**. The dashed horizontal line marked loadings > 0.7 mainly contributing to the component. **(E)** Behavioral tests clustered according to the loading in two components of the PCA.

CB2TG mice

(A)

Variables	Factor loadings	
	Principal component 1 (40.96 %)	Principal component 2 (17.77%)
Persistence to response	0.87	-0.15
Motivation	0.66	0.18
Compulsivity	0.49	0.07
Impulsivity	0.83	-0.21
Cognitive flexibility (errors)	-0.11	-0.68
Appetitive associative learning	-0.05	0.79
Aversive associative learning (errors)	0.86	0.20

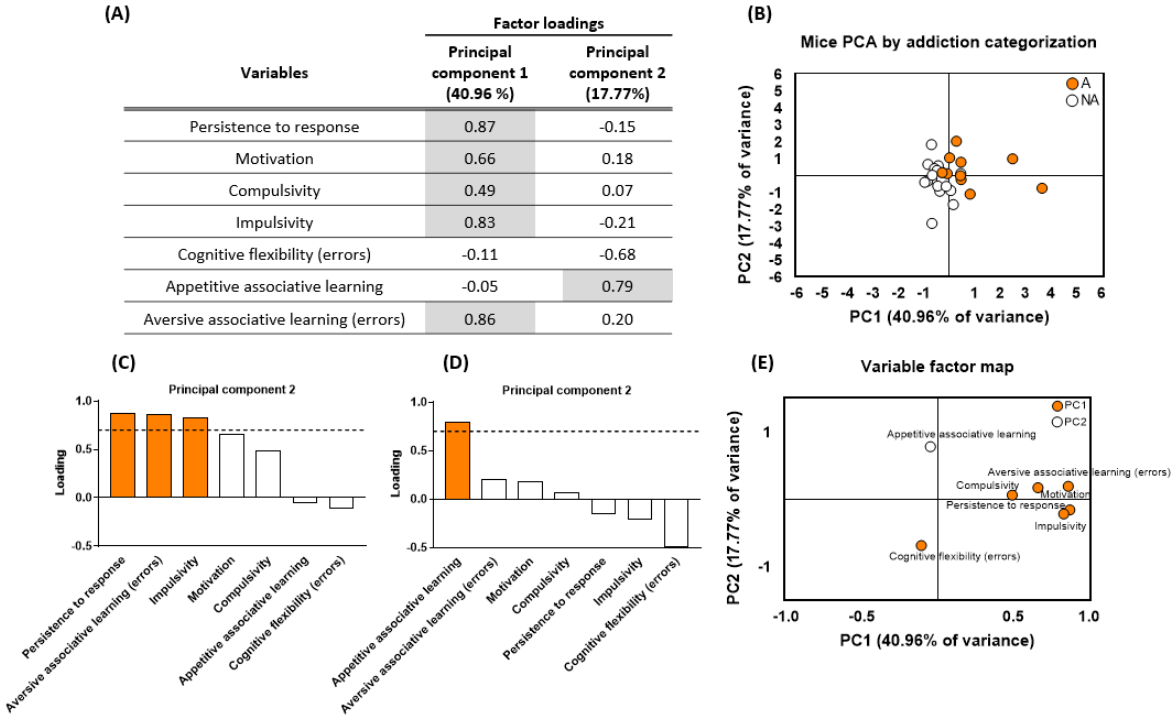


Figure 14. Principal components analysis (PCA) of food addiction phenotype in CB2TG mice. (A) PCA of the three addiction criteria and the four phenotypic traits with factor loadings of principal component (PC) 1 (40.96%) and PC2 (17.77%). All addiction criteria tests, as well as the impulsivity trait and the aversive associative learning, weighted more in PC1. In contrast, appetitive associative learning showed the highest loading in PC2. The cognitive flexibility item did not meet the proposed loading criterion (0.40) (Field, 2018). **(B)** Mice subjects clustered by presence addicted or non-addicted on the space yielded by two components of the PCA that account for the maximum data variance. (n=10 addicted (A) mice, n=18 non-addicted (NA) mice). **(C-D)** Graphs with the order of factor loading of the different variables in the PC1 **(C)** and PC2 **(D)**. The dashed horizontal line marked loadings > 0.7 mainly contributing to the component. **(E)** Behavioral tests clustered according to the loading in two components of the PCA.

Emotional alterations after long-term exposure to palatable pellets

Anxiety-like behavior

The elevated plus maze was used to assess anxiety-like behavior at the end of the experimental protocol, evaluated by the percentage of time spent in the open arms of the maze. Both CB2KO and CB2TG genotypes displayed unaltered anxiety-like behavior when compared to the WT group in the total population (ANOVA, n. s., **Fig. 12A**), as well as when addicted and non-addicted mice were separately analyzed (ANOVA, n. s., **Fig. 12B-C**). In contrast, CB2KO mice showed an increased number of total entries to both open and closed arms in comparison to WT mice in the total population (ANOVA, Bonferroni, $p < 0.05$, **Fig. 12D**). This significant difference was also observed among CB2KO non-addicted mice (ANOVA, Bonferroni, $p < 0.05$, **Fig. 12E**), but no within the addicted group (ANOVA, n. s., **Fig. 12F**). However, the number of total entries was not considered an anxiety-like behavior measurement, but an estimation of locomotor activity, as previously reported (La Porta *et al.*, 2015).

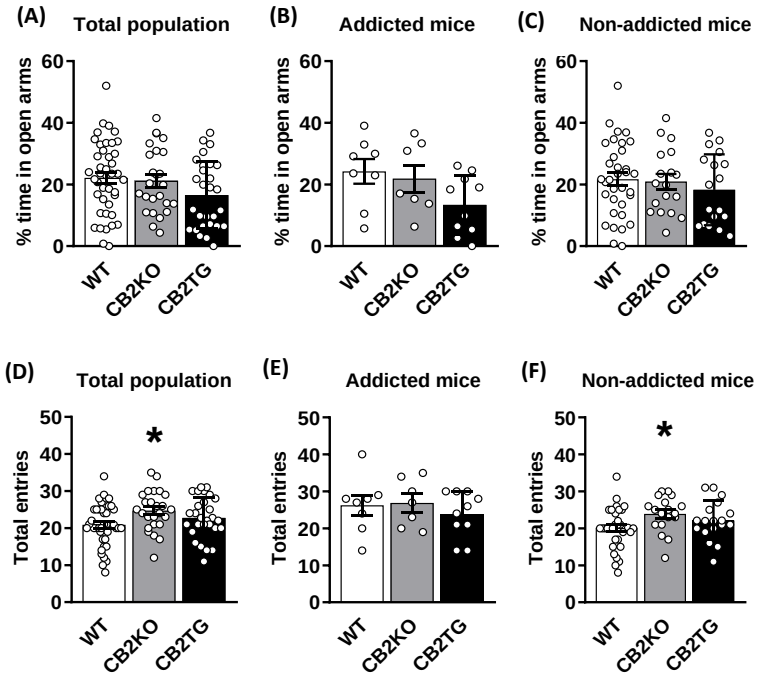


Figure 12. Anxiety-like behavior in mice exposed to long-term operant training maintained by chocolate-favored pellets. Percentage of time spent in the open arms (anxiety-like behavior) and number of total entries to the arms maze (locomotor activity) **(A, D)** the total population (WT n = 42, CB2KO n = 25, CB2TG n = 28), **(B, E)** mice classified as addicted (WT n = 8, CB2KO n = 7, CB2TG n = 10), **(C, D)** mice classified as non-addicted (WT n = 34, CB2KO n = 18, CB2TG n = 18). Both CB2KO and CB2TG mice displayed unaltered anxiety-like behavior when compared to WT mice. Data are expressed as mean ± SEM. * $p < 0.05$ vs. WT (ANOVA, Bonferroni).

Depressive-like behavior

The forced swimming test was used to assess depressive-like behavior at the end of the experimental protocol, evaluated by immobility in the water maze for 4 min. Significant genotype differences were found when the immobility time of the total population was analyzed (U

Mann-Whitney, $p < 0.01$, **Fig. 13A**), since CB2KO mice exhibited less immobility time than WT mice. Moreover, CB2KO mice classified as addicted revealed a decreased time of immobility when compared to WT and CB2TG addicted mice (ANOVA, Bonferroni, $p < 0.05$, **Fig. 13B**), whereas CB2TG addicted mice showed depressive-like manifestations like WT addicted animals (ANOVA, n. s., **Fig. 13B**). Non-significant genotype differences were observed among the non-addicted mice population (ANOVA, n. s., **Fig. 13C**). Therefore, the lack of CB2R in mice results in a protective factor to develop depressive-like behavior after chronic exposure to highly palatable pellets.

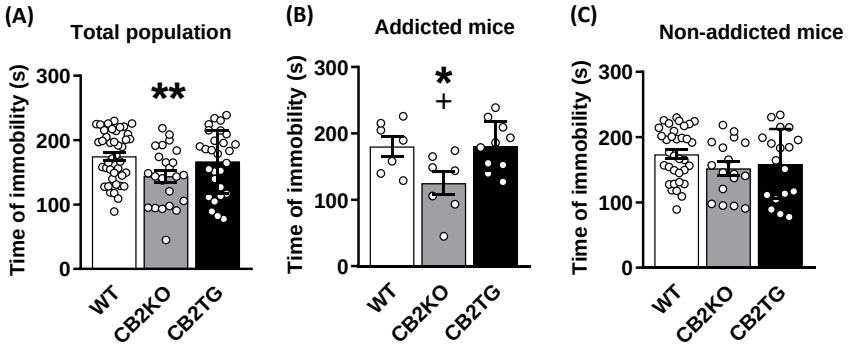


Figure 13. Depressive-like behavior in mice expose to long-term operant training maintained by chocolate-favored pellets. Time of immobility in seconds (s) in the forced swimming test. **(A)** Total population (WT n = 42, CB2KO n = 25, CB2TG n = 28). ** $p < 0.01$ vs. WT (Kruskal-Wallis, U Mann-Whitney) **(B)** Mice classified as addicted (WT n = 8, CB2KO n = 7, CB2TG n = 10). CB2KO mice exhibited less time of immobility in comparison to CB2TG and WT mice. **(C)** Mice classified as non-addicted (WT n = 34, CB2KO n = 18, CB2TG n = 18). Data are expressed as mean ± SEM. * $p < 0.05$ vs. WT, + $p < 0.05$ vs. CB2TG (ANOVA, Bonferroni)

DISCUSSION

The endocannabinoid system (ECS) is a modulatory system that participates in multiple physiological processes, including nociceptive, emotional and rewarding responses (Battista *et al.*, 2012) of particular relevance for the aims of this Doctoral Thesis. Dysregulation of this endogenous system has been related to diverse pathological conditions and is currently covered by classical therapies (Di Marzo, 2018).

The ECS fine-tunes neurotransmitter release of different systems (glutamate, GABA, dopamine, among others) at the central level primarily via CB1R- or CB2R-dependent mechanisms (Kano *et al.*, 2009). Due to its widespread distribution and abundance in the human body, CB1R participates in numerous pathophysiological processes and its genetic and pharmacological modulation has been extensively investigated (Iannotti *et al.*, 2016). However, the anatomical location of CB1R in several brain areas underlies psychoactive, motor, and cognitive effects that limit its therapeutic use (Manzanares *et al.*, 2018). Recent research on CB2R represents an alternative approach to avoid these central side effects since CB2R presence in the brain is lower than CB1R and CB2R is often overexpressed during pathological conditions (Di Marzo, 2018). Therefore, CB2R modulation has great therapeutic potential.

The overall purpose of this Doctoral Thesis was to investigate the role of CB2R and its genetic manipulation in two specific pathological conditions, neuropathic pain and food addiction, to clarify the possible therapeutic interest of this endogenous target. Using relevant mouse

models, we have focused our attention on exploring the contribution of CB2R in several behavioral manifestations of these disorders.

The main results obtained in each of the objectives of the present Doctoral Thesis will be discussed in this section.

Involvement of CB2R in the nociceptive, cognitive, and emotional manifestations of neuropathic pain

The ECS plays an important role in modulating pain responses. Indeed, one of the earliest and widely known uses of the *Cannabis sativa* plant was to treat pain (Huang *et al.*, 2016) and several studies have shown that activation of the cannabinoid receptors modulates nociceptive responses acting at peripheral, spinal, and supraspinal levels. Neuropathic pain is a complex chronic pain condition currently lacking adequate treatment and it is often associated with emotional consequences that difficult the therapeutic approach (Finnerup *et al.*, 2015). A growing body of evidence suggests the interest of the ECS as a potential therapeutic target for neuropathic pain and its emotional comorbidities. The analgesic effect of cannabinoid agonists has been demonstrated in different neuropathic pain animal models and human trials by either CB1R and CB2R activation (Davis, 2014; Maldonado *et al.*, 2016; Shang and Tang, 2017). CB1R and CB2R can also modulate and improve other components of pain perception, including emotional and cognitive comorbidities, acting in cortical and limbic areas (Marco *et al.*, 2011; Poleszak *et al.*, 2018). Despite these promising results, the treatment of neuropathic pain is often limited due to the side effects typically associated with CB1R stimulation and

mainly by the insufficient efficacy of targeting this receptor (Namaka *et al.*, 2009). For that reason, one main objective of this thesis was to study the therapeutic potential of CB2R in a mouse model of neuropathic pain to alleviate the nociceptive and emotional manifestations associated with this chronic condition.

Our first study provides novel findings to clarify the mechanisms involved in the resistant phenotype of Fmr1KO mice, a mouse model of Fragile X syndrome, against the nociceptive, cognitive, and affective manifestations that occur after a peripheral nerve injury. We reveal the participation of CB2R on the protective effects of this phenotype in the development of neuropathic pain and identify specific changes in the endocannabinoid system and the expression of pain-related genes in spinal and supraspinal areas in this mouse model protected against chronic pain manifestations.

Nerve injury induced in our experimental conditions the expected mechanical allodynia and heat hypersensitivity in WT mice (La Porta *et al.*, 2016), whereas both **nociceptive manifestations** were attenuated in Fmr1KO mice during at least 2 weeks after nerve ligation. In agreement, previous studies have reported that Fmr1KO mice failed to show enhanced mechanical and thermal hypersensitivity in response to nerve injury (Price *et al.*, 2007; Wang *et al.*, 2016). As chronic pain is often associated with several functional and emotional consequences that impair the quality of life of patients (Colloca *et al.*, 2017), we also investigated these **comorbid manifestations of neuropathic pain** in Fmr1KO mice. Neuropathic pain impaired long-term memory and produced depressive-like behavior in WT mice, as previously reported

(Chung *et al.*, 2017; Martínez-Navarro *et al.*, 2019). The lack of *Fmr1* gene has been widely related to memory deficits in mice (Gomis-González *et al.*, 2016). Accordingly, Fmr1KO mice showed cognitive impairment in sham and neuropathic pain conditions. Nevertheless, Fmr1KO mice did not develop classical depressive-like behavior associated with nerve injury, suggesting the participation of FRMP in this emotional manifestation of chronic pain.

Fmr1KO mice with a partial deletion of CB2R and their WT littermates were used to evaluate the involvement of this receptor in the neuropathic pain-resistant phenotype displayed by Fmr1KO mice. **CB2R** presence was required to obtain the protective phenotype on nociceptive and emotional responses since the WT phenotype induced by PSNL was rescued when CB2R was partially removed in Fmr1KO mice. In addition, sham mice partially lacking CB2R showed an enhanced depressive-like behavior, suggesting an antidepressant function of CB2R under basal conditions. Accordingly, previous studies targeting CB2R with pharmacological tools or genetic deletion have also shown antidepressant effects of CB2R activation (Liu *et al.*, 2017; Ishiguro *et al.*, 2018). On the other hand, sham mice partially lacking CB2R did not show cognitive deficits under basal conditions, as other studies have reported when this receptor was pharmacologically blocked (Busquets-Garcia *et al.*, 2013). Similarly, CB2R deletion did not modify the cognitive impairment associated with the nerve injury or to the lack of FMRP, ruling out a function of CB2R promoting cognitive impairment. Altogether, these results highlight that CB2R participates in the protective phenotype displayed by Fmr1KO mice against the

nociceptive and emotional manifestations of neuropathic pain and prevents depressive-like behavior in WT mice under basal conditions.

To explore pain-associated biochemical alterations in our experimental conditions, we evaluated the levels of endocannabinoids and related lipids in the spinal cord dorsal horn of WT and Fmr1KO mice. Sciatic nerve injury increased PEA and OEA levels in the spinal cord of Fmr1KO mice, but not in WT mice. This enhanced tone of **spinal N-acylethanolamines** could participate in the attenuation of nociceptive responses observed in Fmr1KO mice after the nerve injury. In agreement, previous studies have shown that increased levels of PEA and OEA alleviate different chronic pain states (Suardíaz *et al.*, 2007; Gugliandolo *et al.*, 2018). On the contrary, neuropathic pain was not associated in our study with altered levels of 2-AG or AEA at the spinal level, neither in WT nor Fmr1KO mice. Previous studies showed that changes of 2-AG and AEA at the spinal level were dependent on time at early periods after nerve injury (from day 3 to 7) (Petrosino *et al.*, 2007), whereas no major changes of endocannabinoids were revealed by other authors at early stages (Starowicz *et al.*, 2013; Thomas *et al.*, 2020). However, previous studies did not investigate the changes in endocannabinoid levels at late periods, such as in our study (21 days after nerve injury).

Taken into account these changes on N-acylethanolamines, we investigated the expression of specific genes related to ethanolamines pathways and pain processing at spinal and supraspinal levels, including *Ppara*, *Rela*, *Grm5,* and *Homer1a*. PPARα, the protein encoded by *Ppara*, is mainly activated by PEA and OEA and is involved

in nociception modulating the activity of pro-inflammatory factors, such as NF-κB, encoded by *Rela* (Iannotti *et al.*, 2016; Di Marzo, 2018). Nerve ligation did not modify *Ppara* and *Rela* gene expression in the **spinal dorsal horn** of WT or Fmr1KO mice. In accordance, previous studies did not reveal alterations in *Ppara* expression in the spinal cord of mice after peripheral nerve injury (Okine *et al.*, 2015). We presume that the absence of alterations in *Rela* expression in our experimental conditions could be explained by the absence of changes in *Ppara* gene expression (Rakhshandehroo *et al.*, 2010).

The mGluR5-Homer1a complex, formed by the proteins encoded by these genes, is implicated in the development of chronic pain and associated negative affective states through glutamate-mediated cellular signaling (Obara *et al.*, 2013a; Chung *et al.*, 2017). The expression levels of *Grm5* and *Homer1a* in the **spinal dorsal horn** of WT or Fmr1KO mice also remained unaltered after nerve injury. Accordingly, no alterations of mGluR5 or Homer1a expression were previously reported following peripheral nerve injury (Obara *et al.*, 2013b; Michot *et al.*, 2017). This absence of expression changes highlights the complex nature of pain processing and prompted us to search for molecular mechanisms in other somatosensory areas that could explain the protective phenotype of Fmr1KO mice.

The expression of *Ppara* and *Rela* in the **mPFC** was enhanced after nerve injury in Fmr1KO, but not in WT mice. This cortical *Ppara* enhancement may potentially prevent the nociceptive manifestations of neuropathic pain in Fmr1KO mice considering the antinociceptive effects reported by PPARα activation (Di Marzo, 2018). Although

PPARα induces antinociceptive effects by preventing upregulation of the protein product of *Rela* (NF-κB) (Iannotti *et al.*, 2016), a previous study demonstrated that PPARα agonist administration did not modify mRNA levels of *Rela* (Nakano *et al.*, 2018). Therefore, *Rela* modifications in Fmr1KO mice could be independent of *Ppara*. On the other hand, Fmr1KO mice exhibited diminished *Grm5* and *Homer1a* expression in the mPFC under sham and neuropathic pain conditions. The complex mGluR5-Homer1a increases glutamatergic input and nociceptive transmission under neuropathic pain conditions (Obara *et al.*, 2013b), and the blockade of cortical mGluR5 decreases mechanical and thermal hypersensitivity after spinal nerve injury (Chung *et al.*, 2017). Hence, the reduced cortical expression of the genes encoding for the pronociceptive mGluR5-Homer1a complex may also contribute to the attenuated nociceptive manifestations of Fmr1KO mice after nerve ligation.

The **amygdala** is a crucial integrator of emotional processing responsible for the affective-motivational dimension of pain. Indeed, neuronal hyperactivity of this limbic area due to sustained nociceptive input trigger anxious and depressive alterations associated with chronic pain (Gonçalves and Dickenson, 2012). In our study, nerve-injured WT mice showed increased *Grm5* expression restricted to the right amygdala, in agreement with previous studies describing the enhanced activity of the right amygdala in chronic pain conditions (Allen *et al.*, 2020). Indeed, previous reports described increased mGlur5 function in the right amygdala of mice subjected to different pain models (Crock et al., 2012; Kolber et al., 2010). Interestingly,

Fmr1KO mice did not show this increase in *Gmr5* expression and, in turn, exhibited decreased *Ppara* expression in this brain area. Previous studies showed that mGluR5 inhibition in the right amygdala decreased nociceptive responses in different chronic pain models (Kolber *et al.*, 2010; Crock *et al.*, 2012), suggesting that the unaltered expression of Grm5 in the right amygdala of Fmr1KO mice could participate in the antinociceptive phenotype observed in these animals. Unexpectedly, nerve ligation induced also significant changes in the left amygdala of Fmr1KO mice revealed by significant downregulations of *Rela* and *Homer1a*. Positive correlations between the expression of the pronociceptive genes *Rela* and *Homer1a* (Obara *et al.*, 2013b; Paterniti *et al.*, 2017) and the time of immobility in the forced swimming test were obtained exclusively in the contralateral left amygdala of WT and Fmr1KO mice. In agreement, preclinical animal models have demonstrated divergent functions of the left and right amygdala (Sadler *et al.*, 2017; Cooper *et al.*, 2018). Thus, our data is compatible with the participation of the left amygdala in the depressive-like phenotype associated with chronic pain conditions, suggesting that decreased expression of *Rela* and *Homer1a* in the contralateral left amygdala could prevent this emotional manifestation of neuropathic pain.

Overall, we showed that CB2R is required to obtain a protective phenotype against the nociceptive and emotional manifestations of neuropathic pain in Fmr1KO mice. Increased spinal levels of PEA and OEA and decreased expression of glutamate-transmission genes at supra-spinal levels are associated with the attenuated nociceptive

responses of Fmr1KO mice. In addition, our findings support the lateralized activity of the left and right amygdala to modulate the affective dimension of chronic pain and highlight the role of glutamate transmission-related genes in this area in the prevention of depressive-like behavior associated with chronic pain conditions. Because depression and chronic pain are often associated (Huang *et al.*, 2016), appropriate treatment of both emotional components and painful symptoms may improve the quality of life of neuropathic pain patients.

Involvement of CB2R in the development of food addiction and its comorbid emotional alterations

In this Thesis, we have also evaluated the involvement of CB2R in another chronic pathological condition that we hypothesized to be closely related to the physiological role played by these receptors in the development of food addiction.

The abusive consumption of highly caloric and processed food in modern societies beyond homeostatic energy requirements has prompted the interest to study the neurobiological mechanisms underlying compulsive eating behaviors (Meule, 2015; Schulte *et al.*, 2015). Palatable food intake, as well as drugs of abuse, stimulates the brain reward system activating the mesocorticolimbic dopaminergic (DA) circuit (Volkow *et al.*, 2010). Prolonged exposure to both kinds of stimuli produces repeated and excessive release of DA within this circuit that may trigger long-lasting neurobiological adaptations in brain areas involved in addictive-like responses (Koob and Volkow, 2016; Moore *et al.*, 2018). Thus, richly fat and carbohydrates diets may

be damaging not only because of the subsequent overweight and associated health risks (Meule *et al.*, 2015), but maladaptations within the reward system could modify the pattern of food intake from controlled to compulsive (Hebebrand *et al.*, 2014; Moore *et al.*, 2017b).

The ECS plays a critical role in these adaptive changes produced by rewarding stimuli (Manzanares *et al.*, 2018). Growing evidence supports the involvement of the ECS in the reinforcing and motivational properties of highly palatable food, mainly through CB1R dependent mechanism (Maccioni *et al.*, 2008). Indeed, long-term daily exposure to palatable food leads to alterations of food-seeking behavior that can be reverted with the administration of the CB1R antagonist rimonabant (Mancino *et al.*, 2015). Whether these behavioral alterations could be due to dysregulations of the glutamatergic or GABAergic inputs in brain regions where CB1R modulates synaptic transmission has been a matter of debate (Lafenêtre *et al.*, 2009). However, it has been recently demonstrated that the lack of CB1R in glutamatergic cortical neurons promotes the release of glutamate in the NAc and prevents the development of food addiction-like behavior generated by palatable food (Domingo-Rodriguez *et al.*, 2020).

In the present study, we aimed to investigate the possible involvement of CB2R in the transition to addiction after repeated seeking of palatable food using a validated mouse model of food addiction (Mancino *et al.*, 2015). Previous studies reported that CB2R might also be associated with addiction vulnerability due to its possible involvement in the modulation of the reward system (Zhang *et al.*, 2021). However, the precise neurobiological mechanisms underlying

vulnerability or resilience to food addiction remain elusive. Our operant behavioral model adopts the DSM-5 and YFAS 2.0 criteria to diagnose drug and food addiction respectively, enabling to categorize mice in vulnerable or resistant extreme populations to develop addictive-like behaviors after chronic exposure to palatable food. We studied the phenotype of food addiction in WT mice, CB2R knockout mice (CB2KO) and mice overexpressing CB2R (CB2TG) during long operant training maintained by chocolate-flavored pellets.

The **lack of CB2R** induced a strong resilience phenotype to food addiction during the early exposure to palatable pellets, as revealed by the significantly reduced percentage (4.0%) of CB2KO addicted mice. Nevertheless, addiction is a chronic disorder where initially goal-directed behavior to seek the reward becomes a deleterious habit formation after extended reward exposure (Luque *et al.*, 2020). Thus, the resilient phenotype displayed by CB2KO mice during the early operant training disappeared after prolonged exposure to highly palatable food, losing the initial protective factor. In contrast, the **overexpression of CB2R** induced an enhanced vulnerability to develop food addiction after chronic exposure to palatable chocolate-flavored pellets, revealed by the significantly increased percentage (35.71%) of addicted mice in this transgenic group.

Contradictory results have been reported about the involvement of CB2R in the rewarding effects of different **drugs of abuse**. Thus, mice lacking CB2R showed attenuation of nicotine-seeking behavior (Navarrete et al. 2013), but increased preference for and vulnerability to ethanol consumption (Ortega-Álvaro et al., 2015). However, a

reduction of cocaine and ethanol self-administration was reported in transgenic mice overexpressing CB2R (Aracil-Fernández *et al.*, 2012) and after administration of the CB2R agonist JWH133 (Navarrete *et al.*, 2018), respectively. Our results using a **natural reward** showed that genetic overexpression of CB2R induced a vulnerable phenotype to develop food addiction after long-term operant training with palatable chocolate-flavored pellets. Interestingly, previous studies have shown that CB2KO mice fed with a high-fat diet present less adipose tissue, obesity-associated inflammation, and insulin resistance in comparison to WT mice under similar experimental conditions (Deveaux *et al.*, 2009; Agudo *et al.*, 2010). Thus, deletion of CB2R seems to be a protective factor in the development of diet-induced obesity in rodents, although CB2KO and WT mice showed similar (Deveaux *et al.*, 2009) or even higher (Agudo *et al.*, 2010) intake of high-fat food.

Our results showed that CB2KO mice obtained a reduced number of **chocolate-flavored pellets** over the entire operant training period suggesting that palatable pellets were less reinforcing for these mutants. This protective phenotype to develop food addiction displayed by CB2KO mice was previously reported in other drugs of abuse, such as nicotine (Navarrete et al. 2013). However, CB2KO mice did not show differences in **body weight** and **locomotor activity** in comparison to WT mice similar to what was previously reported when mutant mice were fed on a high-fat diet for 12 weeks (Alshaarawy *et al.*, 2019). In contrast, CB2TG mice progressively increased the number of reinforcers obtained during the long operant sessions, showing a reduced pellet consumption only during the early training as similarly

exhibited during initial self-administration of other drugs of abuse, such as cocaine (Aracil-Fernández *et al.*, 2012). Despite increasing consumption of chocolate-flavored pellets, these CB2TG mice exhibited a loss of body weight gain in comparison to the WT group over the entire experimental protocol. Previous studies have also described a lean phenotype of mice overexpressing CB2R (Romero-Zerbo *et al.*, 2012) and after a daily administration of the CB2R agonist JWH-015 for 21 days (Verty *et al.*, 2015), highlighting the interest in targeting CB2R in obesity-associated pathologies.

A phenotype of **perseverance** to obtain chocolate-flavored pellets during a period of non-availability of food was also observed in mice overexpressing CB2R during the late period of operant training. The persistence of food-seeking is one core addiction criteria referred to as the loss of inhibitory control over palatable food (Gearhardt *et al.*, 2009). Two complementary frontostriatal circuits have been involved in the loss of inhibitory control in rodents (**Fig. 1**). Neuronal projections from the prelimbic cortex to the NAc (ventral striatum) formed the **Stop circuit** that was reported to be dysregulated during compulsive behaviors (Chen *et al.*, 2013; Hu *et al.*, 2019). In turn, the **Go circuit** is constituted by orbitofrontal cortex-dorsal striatal projections that increased activity when the loss of control was evidenced in murine models of self-administration (Pascoli *et al.*, 2018). Recently, the involvement of the ECS in the loss of inhibitory control over palatable food was demonstrated when the lack of CB1R in prelimbic glutamatergic neurons prevented the development of food addiction-like behaviors (Domingo-Rodriguez *et al.*, 2020). In our experimental

conditions, long-term exposure to palatable food promoted persistent food-seeking behavior in mice overexpressing CB2R. Furthermore, the principal component analysis revealed that the persistence to response loaded more than the motivation and compulsivity traits in the CB2TG phenotype and strong associations were revealed between CB2TG addicted mice and the perseverance to obtain chocolate-flavored pellets. Therefore, we hypothesized that the unbalanced of the Go and Stop circuitries is more pronounced in mice overexpressing of CB2R leading to the maladaptive habit formation in these mice.

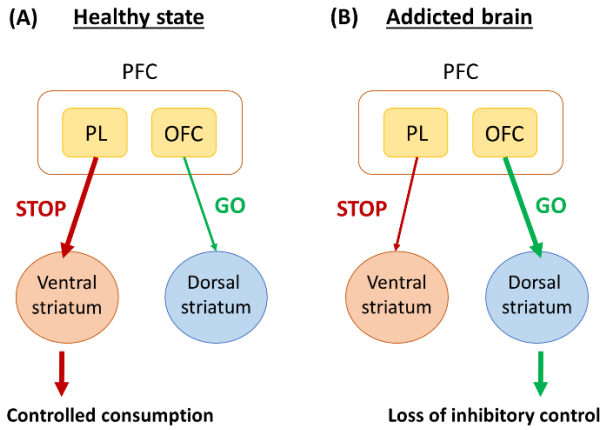


Figure 1. A simplified model of the two murine frontostriatal circuits involved in inhibitory control. (A) In a healthy state, the Stop circuit predominates [red], and automatic responses are suppressed by input from the prelimbic cortex (PL) into the ventral striatum. Thus, an individual in a healthy state exposed to drugs or palatable food prevents excessive intake ("Stop"). **(B)** During addiction, there is an unbalance of the Go and the Stop circuits that overcomes the control. This unbalance is revealed by decreased input from the PL region and enhanced activation of orbitofrontal cortex (OFC) projections into the dorsal striatum. The result is that automatic stimulus-driven behaviors, such as impulsive and compulsive consumption predominate ("Go"). PFC: prefrontal cortex. Adapted from (Goldstein and Volkow, 2011).

In line with the involvement of the ECS in self-control, **impulsive like-behavior** was the phenotypic trait predominantly affected by the lack of CB2R. Thus, **CB2KO mice** showed reduced impulsivity to obtain palatable food over the entire experimental protocol measured by the number of active lever-presses during the time-out period after pellet delivery. Previous publications have confirmed the non-impulsivity trait of CB1KO (Mancino et al., 2015) and Glu-CB1KO mice using different palatable food-seeking tests (Lafenêtre et al., 2009; Domingo-Rodriguez et al., 2020). Moreover, maladaptive impulsivity has been implicated in substance use disorders and antagonists of the CB1R suppressed the impulsivity-related effects of psychostimulant drugs, such as amphetamine (Wiskerke *et al.*, 2011) and nicotine (Wiskerke *et al.*, 2012). However, the role of CB2R in impulsive like-responses is still unknown. Since all drugs of abuse increase DA in the mesolimbic DA system, most clinical studies about addiction have focused on midbrain and basal ganglia structures, which are involved in reward, conditioning, and habit formation (Manzanares *et al.*, 2018). Indeed, data from human brain-imaging studies have demonstrated the involvement of the ventral striatum in impulsive choice (Basar et al., 2010) and CB2R present in DA terminals or resident GABA medium spiny neurons could locally modulate DA release in the **NAc** (Morales and Bonci, 2012). Nevertheless, the NAc is not the only substrate responsible for impulsivity. **PFC** disruption negatively affects a wide range of brain functions, including emotion, cognition, and self-control. Specifically, anterior cingulate (ACC) and orbitofrontal (OFC) cortical regions are implied in inhibitory control since their impairment is associated with a propensity for impulsive and compulsive behaviors

(Volkow *et al.*, 2019). Indeed, results of studies into inhibitory control and drug addiction showed that a deficient Go/No-go task performance was associated with dorsal ACC hypoactivation in cocaine and heroin abusers (Goldstein and Volkow, 2011). Several studies have reported a decreased activity in the PFC, including ACC and OFC areas, in drug abusers (Goldstein and Volkow, 2011; Koob and Volkow, 2016). Therefore, chronic exposure to highly palatable food in our experimental conditions may lead to a PFC hypoactivity in our addicted mice with the ensuing deficient inhibitory control. We hypothesized that CB2R may be involved in the transition from reward-driven to impulsive eating. The presence of CB2R has been confirmed in classical brain circuits relevant in the neurobiology of addiction, including the VTA and NAc (Manzanares *et al.*, 2018), but further studies are required to determine how CB2R modulates DA neurotransmission and find the precise distribution of CB2R within the reward circuit, mainly under these pathophysiological conditions.

Unlike CB2KO mice, long-term exposure to palatable pellets alters the inhibitory control of WT and CB2TG mice, which progressively increased their impulsive-like responses over time. These mice were unable of withholding an anticipated response in comparison to CB2KO mice since they showed enhanced operant responses during the time-out period even when no reward can be obtained. A previous study in rats found that the impulsivity trait increases the risk of developing food addiction-like habits, such as uncontrollable overeating of palatable foods (Velázquez-Sánchez et al., 2015). Our data suggest that prolonged access to palatable food could promote the development of

impulsive-like behaviors and potentially lead to compulsive food intake, especially in those mice overexpressing CB2R, which showed a vulnerable phenotype to food addiction in our experimental conditions. Altogether, the reduced consumption of reinforcers and the reduced impulsive like-behavior to obtain palatable food displayed by CB2KO mice suggest that the lack of CB2R could be a protective factor against impulsive behaviors towards palatable food, as previously reported for CB1R blockade (Mancino *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020).

Food addiction is a complex multifactorial disease that results from the interaction of multiple genes and environmental factors. Not all individuals exposed to palatable food lose control over food intake and develop overeating behaviors. Indeed, genetic vulnerability plays an important role in the evolution of this complex addictive disorder. After repeated seeking of palatable food during 118 sessions, we found that 19.05% of a large cohort of **WT mice** (n = 42) reached the 2-3 criteria and was classified as addicted mice, similar to the prevalence reported in humans (19.9%) using the YFAS food addiction diagnosis (Pursey et al., 2014) and similar to the percentage obtained in previous animal studies (22.2-25%) (Mancino *et al.*, 2015; Domingo-Rodriguez *et al.*, 2020). This percentage of WT addicted mice reflects the vulnerability to develop food addiction in the whole genetically heterogeneous mouse population despite identical exposure to palatable food and highlights the conceptualization of food addiction as a multifactorial disorder. As expected, WT mice reaching 2-3 criteria for palatable food showed the highest responses in the three addiction-like criteria tests,

especially in the perseverance and motivation to obtain palatable food, when compared with WT mice that do not develop addiction (0-1 criteria) in the early, medium, and late periods. In this line, the principal component analysis of WT mice revealed that the persistence to response and motivation tests belong to a different component than the compulsivity test, highlighting the dissociation between tests focused on food-seeking and those focused on compulsive-behaviors. These three food addiction criteria have been classically evaluated in an early and late period (Mancino *et al.*, 2015). In our experimental conditions, CB2KO mice were characterized by a reduced motivation to obtain highly palatable food and by an impaired cue-reactivity to both aversive and appetitive stimuli only in the early period. These significant differences disappeared from the medium period onwards suggesting that CB2R had no major consequences in these associative learning processes between cues and food-taking experience. In contrast, the phenotype of perseverance to obtain highly palatable food observed in CB2TG mice was only observed after long-term operant training. Chronic exposure to addictive substances could lead to long-lasting changes in the circuits underlying rewarding effects (ventral striatum) and habit formation (dorsal striatum) (Everitt and Robbins, 2013). Thus, the medium period rather than the early period constitutes a more insightful timepoint to predict which animal would develop vulnerability or resilience to food addiction in the late period.

Addiction involves a disruption in the cognitive ability to change responding to a previously rewarded stimulus due to hypoactivity of the PFC (Basar *et al.*, 2010; Volkow and Morales, 2015). Indeed,

cocaine-addicted individuals showed negative correlations between accurate responses in a behavioral flexibility test and recent cocaine use (Goldstein *et al.*, 2008). In preclinical studies, drug-addicted mice showed difficulties in changing drug-seeking triggered by stimuli associated with drug delivery (Goldstein and Volkow, 2011). Our results using a natural reward revealed that the genetic disruption or overexpression of CB2R had no major consequences in **cognitive flexibility**, as well as in the **cue-reactivity traits**, maintained by chocolate-flavored pellets. Nevertheless, CB2KO and CB2TG mice did not show learning alterations during the acquisition of the operant training when compared to WT mice.

Drug addiction has been conceptualized as a chronic relapsing disorder characterized by three distinct recurring stages: (1) binge/intoxication phase, (2) emergence of a negative affect when the access to the drug is prevented, and (3) preoccupation/anticipation phase to seek and renewed drug intake (**Fig. 2**) (Koob and Volkow, 2010). The development of the aversive emotional state is known as the **"dark side" of addiction** in which drugs are being taken to prevent or relieve negative states (dysphoria, anxiety, irritability) that result from abstinence or adverse environmental circumstances, such as stress (Koob, 2015). This "dark side" of addiction also seems crucial in the development of food addiction. Initially, consumption of highly palatable foods (high in salt, fat, and sugar) produces feelings of pleasure or gratification (**positive reinforcement**). However, repeated overconsumption of palatable food involves allostatic changes in the brain reward and stress systems that ultimately promote depressive or

anxious responses when those foods are no longer consumed (**negative reinforcement**) (Parylak *et al.*, 2011; Kalon *et al.*, 2016). Consistent with this "dark side" hypothesis, individuals classified as suffering from food addiction by the YFAS have reported significant levels of impairment and distress, such as depressive symptoms, impulsivity, and negative affect (Parylak *et al.*, 2011). On the other hand, patients with psychiatric comorbidity with mood, anxiety, and depression have a high risk to engage in food addiction (Benzerouk *et al.*, 2018; Oliveira *et al.*, 2020).

Considering this evidence, the next step after the chronic daily exposure to palatable food in our experimental sequence was to evaluate the **emotional state** of the different mice genotypes. Our findings suggest a possible involvement of CB2R in depressive-like, but not in anxiety-like manifestations associated with the chronic exposure to palatable food. Indeed, the lack of CB2R resulted in a protective phenotype against the depressive-like behavior since CB2KO mice displayed a reduced time of immobility in the FST. Interestingly, this protection was observed among the CB2KO mice classified as addicted, but not in the animals classified as non-addicted, suggesting that chronic overconsumption of palatable food led to this behavioral change. We discarded associations between the decreased time of immobility of CB2KO mice in the FST and deficits in locomotor activity since these mutants showed an enhanced number of total entries in the arms of the EPM when compared to WT mice. This number of total entries is considered an estimation of locomotor activity, as previously reported (La Porta *et al.*, 2015). In contrast, although a depression-

resistant phenotype was reported in mice overexpressing CB2R in physiological conditions (García-Gutiérrez *et al.*, 2010), the long-term exposure to highly palatable food abolish this protective behavior in CB2TG mice in our experimental conditions, probably due to allostatic changes in the brain reward and stress systems.

Previous animal studies have extensively reported the involvement of CB1R in affective responses (Umathe *et al.*, 2011; Poleszak *et al.*, 2018). Although several publications have reported the involvement of CB2R in the pathophysiology of depression, the results are not consistent. Both CB2R agonists (Hu *et al.*, 2009) and antagonists (García-Gutiérrez *et al.*, 2010) have the potential to induce antidepressant-like effects depending on the experimental design, whereas CB2R deficiency produced anxiogenic-like responses in the light-dark box and elevated plus-maze tests, and a depressogenic-like phenotype in the tail suspension test (Ortega-Alvaro et al., 2011). Similar to CB2R, CB1KO mice develop long-lasting depression and anxiety-related behaviors (Rácz *et al.*, 2015). In our experimental conditions, both CB2KO and CB2TG genotypes displayed unaltered anxiety-like behavior when compared to the WT group. The lack of significant differences in the anxiety-like behavior suggests that the genetic modification of CB2R has no major effect in this domain after chronic exposure to palatable food.

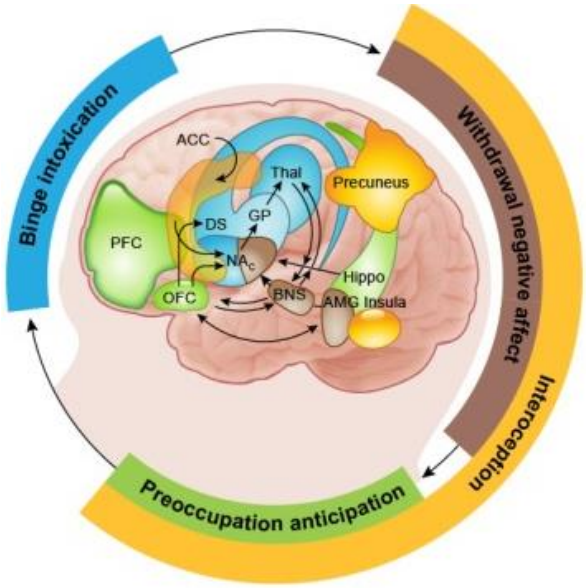


Figure 2. Model of interacting circuits in which disruptions contribute to compulsive-like behaviors underlying drug addiction. The neurocircuitry model has three different functional domains: binge/intoxication involved in rewarding effects: basal ganglia (DS: dorsal striatum, NAc: nucleus accumbens, GP: globus pallidus) and thalamus (Thal) [blue]; withdrawal/negative affect involved in negative emotional states and stress: the extended amygdala (AMG: amygdala, BNS: bed nucleus of the stria terminalis, NAc shell) [brown]; and preoccupation/anticipation involved in craving, impulsivity, and executive function: prefrontal cortex (PFC), orbitofrontal cortex (OFC), and hippocampus (Hippo) [green]. Anterior cingulate cortex (ACC), insula, and precuneus constitute areas of interception between domains [yellow]. Arrows depict major circuit connections between domains. Extracted from (Volkow *et al.*, 2019).

Several areas are involved in the control of anxiety and depressive behaviors, although the amygdala seems to play a prominent role in anxiety-related responses (Mineur *et al.*, 2018). Particularly, the **extended amygdala** (central nucleus of the amygdala, bed nucleus of the stria terminalis, and NAc shell) (Shaded brown areas, **Fig. 2**) has

been shown to have a key role in associated emotions, including the emotional component of pain processing (Neugebauer, 2015) and “the dark side of addiction” (Koob, 2013). Thus, neuroimaging studies revealed differing activation patterns in the extended amygdala of individuals with food addiction compared to healthy controls (Koob, 2021). Other human neuroimaging studies and complementary animal experiments have also identified corticolimbic areas classically related to the reward system involved in the emotional dimension of chronic pain, such as the mPFC, NAc, amygdala, and ACC (Vachon-Presseau et al., 2016). Thereby, the ACC generates affective and motivational pain and addiction-like responses via projections to the amygdala, NAc and mPFC highlighting the high comorbidity between chronic pain, addiction and depression (Thompson and Neugebauer, 2019; Koob, 2021).

Together, our data indicate that CB2R disruption provides substantial protection against the depressive, but not the anxiety manifestations associated with chronic exposure to palatable food (Parylak et al., 2011). Since the CB2R is expressed in brain regions relevant in the neurobiology of addiction, such as the VTA, NAc, and amygdala (Manzanares *et al.*, 2018), targeting CB2R modulation in the extended amygdala would be a promising approach also to attenuate the negative reinforcement in food addiction.

Concluding remarks

This doctoral thesis is focused on the involvement of the ECS through CB2R in the pathophysiology of neuropathic pain and food addiction and their emotional comorbidities. Despite its low expression levels in the CNS, we have provided more evidence supporting that CB2R participates in the development of these complex behaviors.

In our **first study**, we showed that the pain-resistant phenotype of Fmr1KO mice against the nociceptive and emotional manifestations triggered by persistent nerve damage requires the participation of the CB2R. These data highlight the potential interest of targeting CB2R for neuropathic pain treatment since its low presence in neuronal cells (Shang and Tang, 2017) could avoid the psychoactive side effects of cannabinoid drugs (Davis, 2014). Epigenetic studies can help to clarify gene regulation involved in the development of chronic pain, but additional multidisciplinary studies more closely related to the human pain experience, such as high-resolution brain imaging or electrophysiological tools may also be performed to explore the potential use of CB2R ligands as adequate analgesic tools.

In our **second objective**, we demonstrated that CB2R participates in the neurobiological substrate underlying the behavioral and emotional alterations that arise from food addiction, although the precise CB2R circuits involved must be still clarified. The interpretation of findings obtained in mice genetically modified either by removing or increasing CB2R may be restricted by the fact that this genetic modification could promote allostatic changes which may be involved in the effects

evaluated in this genetic model. In this context, it is important to note that the CB2R is expressed in brain immune cells and, thus, an immune cell-neuron interaction might be accountable for the behavioral responses observed in CB2R mutant mice. To this end, further investigations using cell-type-specific deletions of the gene encoding CB2R are required. In addition, pharmacological studies using selective ligands of CB2R would be useful to confirm the relevance of the present results. The absence of female mice in all the studies is a limitation of this thesis since it did not rule out gender discrepancies in these findings.

To sum up, the results of this thesis provide compelling evidence to develop alternative strategies targeting CB2R for the clinical management of both neuropathic pain and food addiction disorders and their co-morbid emotional manifestations.

CONCLUSIONS

The results obtained in the present Doctoral Thesis allow us to draw the following conclusions:

1. CB2R is required to obtain the protective phenotype displayed by Fmr1KO mice against the nociceptive and emotional manifestations of neuropathic pain.

2. CB2R presence prevents depressive-like behavior in WT mice under non-pathological conditions.

3. Increased spinal levels of PEA and OEA, but not of 2-AG or AEA, highlight the possible involvement of N-acylethanolamines in the attenuated nociceptive responses of Fmr1KO mice.

4. Peripheral nerve damage induced changes in the RNA profile at supra-spinal levels. Fmr1KO mice showed decreased expression of pronociceptive glutamate-transmission genes and increased expression of PPARα gene in the mPFC after nerve injury.

5. Decreased expression of pronociceptive genes in the left amygdala of Fmr1KO mice after nerve injury could prevent neuropathic pain-induced depressive-like behavior.

6. Our gene expression studies allowed the identification of genes involved in the pathophysiology of neuropathic pain and supported the lateralized activity of the amygdala to modulate the nociceptive and affective dimensions of chronic pain.

7. The overexpression of CB2R enhanced the vulnerability to develop food addiction promoted by long-term operant training with palatable chocolate-flavored pellets.

8. In contrast, the lack of CB2R is a protective factor against the reinforcing effects and the impulsive-like behaviors towards highly palatable food.

9. Genetic disruption of CB2R provides substantial protection against the depressive manifestations, but not the anxiety-like behavior revealed in addicted individuals after chronic exposure to palatable food.

10. Targeting CB2R modulation could be a promising alternative for the clinical management of both neuropathic pain and food addiction disorders and their co-morbid emotional manifestations.

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ANNEX

Article #1

Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain.

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Abstract

Background: Neuropathic pain is a complex condition characterized by sensory, cognitive and affective symptoms that magnify the perception of pain. The underlying pathogenic mechanisms are largely unknown and there is an urgent need for the development of novel medications. The endocannabinoid system modulates pain perception and drugs targeting the cannabinoid receptor type 2 (CB2) devoid of psychoactive side effects could emerge as novel analgesics. An interesting model to evaluate the mechanisms underlying resistance to pain is the fragile X mental retardation protein knockout mouse (Fmr1KO), a model of fragile X syndrome that exhibits nociceptive deficits and fails to develop neuropathic pain.

Methods: A partial sciatic nerve ligation was performed to wild-type (WT) and Fmr1KO mice having (HzCB2 and Fmr1KO-HzCB2, respectively) or not (WT and Fmr1KO mice) a partial deletion of CB2 to investigate the participation of the endocannabinoid system on the pain-resistant phenotype of Fmr1KO mice.

Results: Nerve injury induced canonical hypersensitivity in WT and HzCB2 mice, whereas this increased pain sensitivity was absent in Fmr1KO mice. Interestingly, Fmr1KO mice partially lacking CB2 lost this protection against neuropathic pain. Similarly, pain-induced depressive-like behaviour was observed in WT, HzCB2 and Fmr1KO-HzCB2 mice, but not in Fmr1KO littermates. Nerve injury evoked different alterations in WT and Fmr1KO mice at spinal and supra-spinal levels that correlated with these nociceptive and emotional alterations.

Conclusions: This work shows that CB2 is necessary for the protection against neuropathic pain observed in Fmr1KO mice, raising the interest in targeting this receptor for the treatment of neuropathic pain.

Significance: Neuropathic pain is a complex chronic pain condition and current treatments are limited by the lack of efficacy and the incidence of important side effects. Our findings show that the pain-resistant phenotype of Fmr1KO mice against nociceptive and emotional manifestations triggered by persistent nerve damage requires the participation of the cannabinoid receptor CB2, raising the interest in targeting this receptor for neuropathic pain treatment. Additional multidisciplinary studies more closely related to human pain experience should be conducted to explore the potential use of cannabinoids as adequate analgesic tools.

KEYWORDS

cannabinoid receptor type-2, depressive-like behaviour, Fmr1KO mice, N-acylethanolamines, neuropathic pain, nociception, protective phenotype

1 | INTRODUCTION

Neuropathic pain is defined as an unpleasant sensory and emotional experience initiated by a lesion or disease of the somatosensory nervous system, mainly associated with spontaneous pain, hyperalgesia and allodynia (Scholz et al., 2019). Neuropathic pain patients often experience anxiety, depression and impaired cognitive functions that diminish their life quality (Descalzi et al., 2017). This clinical entity that affects millions of people worldwide (Colloca et al., 2017) has pathogenic mechanisms that remain largely unknown and current treatments are limited by the lack of efficacy and important side effects (Bouhassira & Attal, 2018). Therefore, there is an urgent need to develop novel therapeutic strategies to improve the quality of life of neuropathic pain patients.

The fragile X mental retardation protein (FMRP), required for the adequate development of neuronal connections, has raised the new interest to clarify pain processing (Aloisi et al., 2017; Busquets-Garcia et al., 2014). The absence of FMRP in humans causes the most common monogenic condition of autistic spectrum disorders, the fragile X syndrome and a prominent feature of this disorder is self-injurious behaviour associated with an alteration of the nociceptive system (Peebles & Price, 2012). Likewise, the preclinical mouse model of fragile X syndrome, a knockout mouse lacking FMRP (Fmr1KO), shows profound deficits in nociceptive sensitisation during neuropathic pain (Price et al., 2007). Therefore, the pain-resistant phenotype of the Fmr1KO mouse represents an appropriate model to investigate the mechanisms underlying these nociceptive deficits and its comorbid manifestations, including cognitive impairment and depressive-like behaviour.

The endocannabinoid system is involved in several physiological processes including affective, cognitive and nociceptive functions (Donvito et al., 2018; Rácz et al., 2015). Cannabinoid receptors type 1 (CB1) and type 2 (CB2) are distributed in main central and peripheral nervous system areas involved in pain processing, such as medial prefrontal cortex (mPFC), amygdala and spinal cord (La Porta et al., 2015). The activation of both receptors reduces nociception and affective alterations produced by neuropathic pain (Klauke et al., 2014; Rácz et al., 2015). We have previously demonstrated that the pain-resistant phenotype of Fmr1KO mice in a model of inflammatory pain depends on CB1 presence (Busquets-Garcia et al., 2013). However, due to its widespread brain distribution, CB1 activation leads to important psychoactive, motor and cognitive effects, which represent major limitations for chronic pain treatment (Davis, 2014). Alternative approaches have been developed to overcome this problem by targeting CB2 that is predominantly expressed in peripheral immune cells, though it is also present at low levels in neurons (Shang & Tang, 2017). Therefore, CB2 could be a potential therapeutic target for neuropathic pain treatment avoiding the risk of centrally mediated side effects.

The aim of this study was to elucidate the neurobiological mechanisms involved in the pain-resistant phenotype of Fmr1KO mice in order to identify potential pharmacological targets for neuropathic pain. For this purpose, we evaluated the nociceptive, cognitive and affective manifestations associated with a peripheral nerve injury in Fmr1KO mice partially lacking CB2 to explore the therapeutic interest of this receptor for neuropathic pain.

2 | METHODS

2.1 | Animals

Taken into account that fragile X syndrome predominantly occurs in male individuals (Razak et al., 2020), all experiments were performed in male mice between 8 and 20 weeks of age. WT ($Fmr1^{+/y}$, $Cnr2^{+/+}$), Fmr1KO ($Fmr1^{-/y}$, $Cnr2^{+/+}$), WT heterozygous for CB2 (HzCB2) ($Fmr1^{+/y}$, $Cnr2^{+/-}$) and Fmr1KO heterozygous for CB2 (Fmr1KO-HzCB2) ($Fmr1^{-/y}$, $Cnr2^{+/-}$) littermates in C57BL/6J genetic background were used. The behavioural experiments were conducted in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). Mice were group-housed (2–4 animals) and maintained in a controlled temperature (21 ± 1°C) and humidity (55 ± 10%) environment. Food and water were available ad libitum and mice were handled during the light phase of a 12 hr light/dark cycle (light on at 8:00 a.m., light off at 8:00 p.m.). All behavioural experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive (2010/63/EU). All the experiments were performed under blind and randomized conditions.

2.2 | Neuropathic pain induction

Mice underwent a partial sciatic nerve ligation (PSNL) at mid-thigh level to induce neuropathic pain, as previously

described (Malmberg & Basbaum, 1998) with minor modifications. Briefly, mice were anesthetized with isoflurane (induction, 5% V/V; surgery, 2% V/V) in oxygen and the sciatic nerve was exposed at the level of the mid-thigh of the right hind leg. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33%–50% of the cranial side of the sciatic nerve using a 9–0 non-absorbable virgin silk suture (Alcon Cusí SA, Barcelona, Spain) and leaving the rest of the nerve untouched. The muscle was then stitched with 6–0 silk (Alcon Cusí) and the skin incision was closed with wound clips. Sham-operated mice underwent the same surgical procedure except that the sciatic nerve was not ligated.

2.3 | Nociception

Sensitivity to mechanical and heat stimuli was used as nociceptive measures of neuropathic pain. Ipsilateral and contralateral hind paw withdrawal thresholds were evaluated the day before, 3, 7 and 14 days after the nerve injury. Mechanical allodynia was quantified by measuring the withdrawal response to von Frey filament stimulation through the up–down paradigm, as previously reported (Chaplan et al., 1994). Filaments equivalent to 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g were used, applying first the 0.4 g filament and increasing or decreasing the strength according to the response. The filaments were bent and held for 5 s against the surface of the hind paws. Heat sensitivity was assessed by recording the hind paw withdrawal latency in response to radiant heat applied with the plantar test apparatus (Ugo Basile, Varese, Italy) as previously reported (Hargreaves et al., 1988). Clear paw withdrawal, shaking or licking was considered a nociceptive response.

2.4 | Cognitive performance

The novel object-recognition test was performed on day 12 after the surgery as previously described (Puighermanal et al., 2009). Briefly, mice were habituated to a V-shaped maze for 9 min on day 1. The following day, mice were introduced in the maze, where two identical objects (familiar objects) were presented in the extremes of the maze. For the memory test performed on the third day, one of the familiar objects was replaced with a new object (novel object) and the total time spent exploring each of the two objects (novel and familiar) was measured. Object exploration was defined as the orientation of the nose towards the object at a distance of <1 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time exploring both objects: (DI = (Tn − Tf)/(Tn + Tf)). A higher discrimination index is considered to reflect greater memory retention for the familiar object. Mice that explored <10 s both objects were excluded from the analysis.

2.5 | Depressive-like behaviour

Depressive-like behaviour was evaluated 19 days after the surgery using the forced swimming test (Porsolt and Bertin, 1977). Briefly, mice were individually placed into a glass cylinder (17.5 × 12.5 cm) filled 15 cm high with water (22 ± 1°C). Mice were subjected to forced swimming for 6 min and the total duration of immobility, disregarding small hind limb movements to keep the head above water, was measured during the last 4 min when mice show a sufficiently stable level of immobility.

2.6 | Endocannabinoid quantification

Animals were sacrificed at the end of the experimental protocol and L3–L5 ipsilateral spinal cord dorsal horns were freshly dissected. Samples were rapidly frozen and stored at −80°C. The quantification of endocannabinoids and related compounds was based on the methodology previously described in plasma (Pastor et al., 2014), adapted for the extraction of endocannabinoids from spinal tissue. The following endocannabinoids and related compounds were quantified: 2-arachidonoyl glycerol (2-AG), N-arachidonoylethanolamine (AEA), palmitoylethanolamide (PEA) and oleoylethanolamine (OEA). Frozen spinal cords (4 ± 1 mg) of mice were placed in a 1 ml Wheaton glass homogenizer and spiked with 25 µl of a mix of deuterated internal standards dissolved in acetonitrile. The mix contained 5 ng/ml of each compound. All internal standards were purchased from Cayman Chemical (Ann Arbor). Tissues were homogenized on ice with 700 µl a mixture of 50 mM Tris-HCl buffer (pH 7.4): methanol (1:1) and the homogenates were transferred to 12 ml glass tubes. The homogenizer was washed twice with 0.9 ml of the same mixture and the contents were combined into the tube giving an approximate volume of 2.5 ml of homogenate. The homogenates were kept on ice until organic extraction to minimize the ex vivo generation of endocannabinoids. Next, homogenates were extracted with 5 ml of chloroform over 20 min by placing the tubes in a rocking mixer. Tubes were centrifuged at 1,700 g over 5 min at room temperature. The lower organic phase was transferred to clean glass tubes, evaporated under a stream of nitrogen in a 39°C water bath and extracts were reconstituted in 100 µl of mixture water: acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to high-performance liquid chromatography

vials with glass micro-vials. Endocannabinoids were separated using an Agilent 6,410 Triple Quadrupole Liquid Chromatography equipped with a 1,200 series binary pump, a column oven and a cooled autosampler (4°C). Chromatographic separation was carried out with a Waters C18-CSH column (3.1 × 100 mm, 1.8 μm particle size) maintained at 40°C with a mobile phase flow rate of 0.4 ml/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. Endocannabinoids and related compounds were separated by gradient chromatography. The ion source was operated in the positive electrospray mode. The selective reaction monitoring mode was used for the analysis. Quantification was performed by isotope dilution with the response of the deuterated internal standards and data were expressed as a percentage of the control group (WT sham).

2.7 | Gene expression analysis

Animals were sacrificed at the end of the experimental protocol and mPFC, amygdala (left and right, separately) and spinal ipsilateral dorsal horns were freshly dissected. The samples were rapidly placed in individual tubes and stored at −80°C. Total RNA was isolated from frozen samples with RNeasy Micro kit (7,004, Qiagen, Stockach, Germany) and subsequently reverse-transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time-Polymerase Chain Reaction (RT-PCR) was carried out in triplicate with a QuantStudio 12K Flex RT-PCR System (4471134, Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (04707516001, Roche, Basel, Switzerland). The expression of the following genes was analysed: nuclear factor NF-Kappa-B P65 subunit (*Rela*), glutamate metabotropic receptor 5 (*Grm5*), homer scaffolding protein 1a (*Homer1a*) and peroxisome proliferator-activated receptor alpha (*Ppara*). Levels of the target genes were normalized against the housekeeping gene beta-2-microglobulin (*B2m*) and compared using the ΔΔCt method (Livak & Schmittgen, 2001). The following specific primers were used: 5′-CTTCCTCAGCCATGGTACCTCT-3′ (*Rela* forward); 5′-CAAGTCTTCATCAGCATCAAACTG-3′ (*Rela* reverse), 5′-GTCCTGGCCCACTGACGA-3′ (*Grm5* forward); 5′-GGTCACCCCATCGAAGATAC-3′ (*Grm5* reverse), 5′-GGGAGGATGGAGACACAGC-3′ (*Homer1a* forward); 5′-CGGTCCGTCCCTTTTTCCTT-3′ (*Homer1a* reverse), 5′-AGAGGGCTGAGCGTAGGTAA-3′ (*Ppara* forward); 5′-ATTGGGCCGGTTAAGACCAG-3′ (*Ppara* reverse), 5′-TTCTGGTGCTTGTCTCACTGA-3′ (*B2m* forward); 5′-CAGTATGTTCGGCTTCCCATTC-3′ (*B2m* reverse).

2.8 | Statistics

All the data were first subjected to a Shapiro-Wilk test of normality. The time course of nociceptive thresholds was analysed using a linear mixed model with three factors (surgery, genotype, time and their interactions) considering the presence of non-Gaussian distribution in some of the experimental days. Bonferroni *post hoc* analysis was performed when pertinent. Long-term memory and endocannabinoid quantification and RT-PCR data were analysed with a two-way ANOVA (genotype, surgery) followed by Bonferroni *post hoc*. Depressive-like behaviour and endocannabinoid quantification and RT-PCR data were analysed with a Kruskal–Wallis followed by U Mann Whitney with Bonferroni adjustment for multiple comparisons taken into account that these data did not follow a normal distribution. A probability of 0.05 or less was considered statistically significant. IBM SPSS 19 (SPSS Inc., Chicago, USA) was used to analyse the data. Detailed statistical analysis is presented in Supplementary Tables S1-S4.

3 | RESULTS

3.1 | Role of CB2 in the nociceptive manifestations of neuropathic pain in Fmr1KO mice

Nociceptive sensitivity to mechanical and heat stimulation was evaluated under basal conditions and 3, 7 and 14 days after PSNL or sham surgery (Figure 1).

3.1.1 | Mechanical allodynia

The von Frey test was used to assess sensitivity to mechanical stimuli in sham and neuropathic pain conditions. All mice showed similar nociceptive thresholds in naïve conditions in both ipsilateral and contralateral paws (Figure 2a). After surgery, nerve-injured animals developed mechanical allodynia, as revealed by significant differences between ipsilateral and contralateral paw sensitivity. As expected, Fmr1KO mice revealed decreased mechanical allodynia following nerve injury when compared to WT mice. Interestingly, the WT mice phenotype on the mechanical nociceptive responses was rescued when CB2 was partially removed in Fmr1KO mice (Figure 1a). No significant differences in mechanical thresholds following sham surgery were observed between WT and the other genotypes (Figure 2a). Therefore, the deletion of the Fmr1 gene decreases the mechanical allodynia associated with the nerve injury and CB2 is involved in this phenotype.

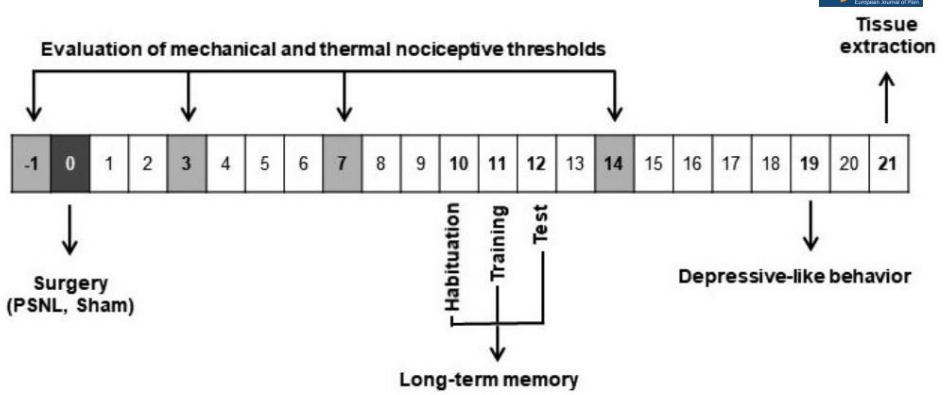

FIGURE 1 Experimental procedure. Baseline mechanical and heat nociceptive thresholds were measured the day before PSNL or sham surgery. Mechanical and heat nociception were assessed again on days 3, 7 and 14 after the surgery. Cognitive performance and affective behaviour were also evaluated under sham and neuropathic pain conditions. Long-term memory was evaluated on day 12 and depressive-like behaviour was assessed on day 19 after the surgery. Mice were sacrificed 21 days after the surgery and tissue collection was performed for molecular analysis

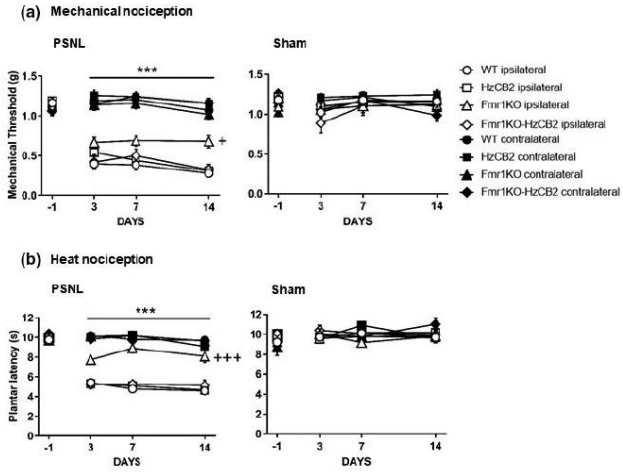

FIGURE 2 Nociceptive sensitivity to mechanical and heat stimulation in sham and neuropathic pain conditions. Mice were tested on the ipsilateral and contralateral paws to evaluate mechanical allodynia (mechanical thresholds in grams) in the von Frey test and heat hyperalgesia (plantar latency in seconds) in the plantar test under basal conditions and on days 3, 7 and 14 after PSNL or sham surgery. (a) The development of mechanical allodynia after nerve injury mice was demonstrated by significant differences between contralateral and ipsilateral paw sensitivity, which was significantly attenuated on the ipsilateral paw of Fmr1KO mice. No differences in mechanical thresholds following sham surgery were observed between the different genotypes. (b) Heat hyperalgesia after nerve injury was also confirmed by significant differences between contralateral and ipsilateral paw withdrawal latencies. Fmr1KO mice also showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery in comparison to WT mice. No differences in heat hypersensitivity following sham surgery were observed between the different genotypes. Data are expressed as mean ± *SEM* ($n = 13–19$ per group). ***$p < .001$ between contralateral and ipsilateral paws; +$p < .05$, +++$p < .001$ versus WT ipsilateral paw (Linear mixed model, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S1

3.1.2 | Heat hyperalgesia

Sensitivity to thermal stimuli in the plantar test was also used as a nociceptive measure of neuropathic pain. All mice showed similar withdrawal latencies of both ipsilateral and contralateral paws in naïve conditions (Figure 2b). The nerve injury induced heat hyperalgesia in all genotypes in comparison to the contralateral paw, but this nociceptive behaviour

was significantly attenuated on Fmr1KO mice. These mutants showed decreased heat sensitivity on the ipsilateral paw following PSNL-surgery compared to WT littermates. When CB2 was partially removed from Fmr1KO mice (Fmr1KO-HzCB2 mutants), thermal hypersensitivity was similar to WT mice (Figure 2b). Sham surgery did not alter the plantar withdrawal latencies of any group (Figure 2b). Thus, the deletion of the Fmr1 gene decreases the heat hypersensitivity associated with the nerve injury and CB2 is also involved in this phenotype.

3.2 | Role of CB2R in memory and emotional alterations associated with neuropathic pain in Fmr1KO mice

Cognitive performance and depressive-like behaviour were evaluated under sham and neuropathic pain conditions 12 and 19 days after the surgery, respectively (Figure 1).

3.2.1 | Cognitive performance

The novel object recognition test was used to assess long-term memory after the induction of neuropathic pain or sham surgery. Long-term memory impairment was revealed in WT and HzCB2 mice exposed to PSNL by a decrease in the discrimination index in comparison to sham littermates, whereas no significant effect was observed in Fmr1KO and Fmr1KO-HzCB2 mice. These mutants already showed a low discrimination index compared to WT mice regardless the surgery (Figure 3a). Hence, both nerve injury and lack of Fmr1 gene produce cognitive impairment that is not modified by the partial deletion of CB2.

3.3 | Depressive-like behaviour

The forced swimming test was used to assess depressive-like behaviour associated with neuropathic pain. As expected, nerve injury induced depressive-like behaviour revealed by a significant increase in the time of immobility of WT and HzCB2 animals in comparison to sham-operated mice. Interestingly, HzCB2 mutants subjected to the sham surgery showed increased depressive-like behaviour when compared to WT sham mice, suggesting a possible anti-depressive effect of CB2. Moreover, Fmr1KO mice did not develop depressive-like behaviour associated with the PSNL surgery and showed different responses to nerve-injured WT and HzCB2 mice. However, the depressive-like behaviour was rescued in Fmr1KO mutants partially lacking CB2 (Figure 3b). Therefore, the inhibition of depressive-like behaviour in Fmr1KO mice depends on CB2 presence, which also modifies this behaviour under basal conditions.

3.4 | Effects of nerve injury on modulating the endocannabinoid tone at spinal cord level

To explore the possible mechanisms underlying the effects of Fmr1 deletion on the nociceptive manifestations

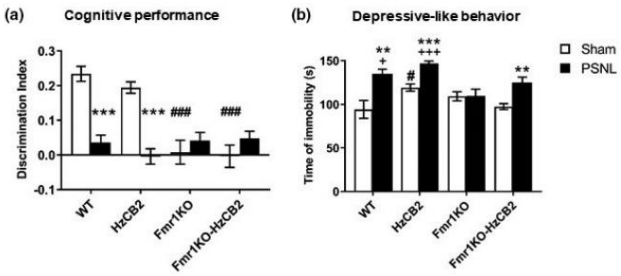

FIGURE 3 Cognitive and emotional behaviours in mice exposed to sham or PSNL surgery. Long-term memory impairment (discrimination index in the novel recognition test) and depressive-like behaviour (time of immobility in seconds in the forced swimming test) were evaluated 12 and 19 days after the induction of the neuropathy, respectively. (a) Nerve injury impaired memory significantly in WT and HzCB2 mice compared to sham mice, whereas Fmr1KO and Fmr1KO-HzCB2 mice show a low discrimination index regardless of the surgery. (b) PSNL significantly increased the depressive-like behaviour in WT, HzCB2 and Fmr1KO-HzCB2 mice, but not in Fmr1KO mice, as indicated by the time of immobility. HzCB2 mice showed a pro-depressive phenotype under sham conditions in comparison to WT sham animals. Data are expressed as mean ± *SEM* ($n = 11–22$ per group). Novel object recognition test: ***p <.001 versus Sham mice of each genotype; ###p <.001 versus WT sham (ANOVA, Bonferroni test). Forced swimming test: *p <.05, **p <.01, ***p <.001 versus Sham mice of each genotype; #p <.05 versus WT sham; +p <.05, +++p <.001 versus Fmr1KO PSNL mice. (Kruskal–Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S2

of neuropathic pain, we evaluated the levels of endocannabinoids and related lipids in the spinal cord dorsal horn of WT and Fmr1KO mice. 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (AEA) are the main endogenous ligands of CB1 and CB2, whereas palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) belong to the N-acylethanolamines family and display anti-inflammatory properties that could participate in the protective phenotype of Fmr1KO mice mainly through peroxisome proliferator-activated receptors (PPARs) (Di Marzo, 2018). Nerve injury did not change the expression of 2-AG, AEA, PEA or OEA in WT mice compared to sham littermates at the spinal level (Figure 4a,b). In contrast, nerve-injured Fmr1KO mice showed increased levels of PEA and OEA, but not 2-AG or AEA when compared to sham mutants (Figure 4c,d). Sham-operated Fmr1KO mice did not show significant alterations in any of these endocannabinoids in comparison to control WT mice (Figure 4a-d). These findings reveal an association between N-acylethanolamines levels and the nociceptive changes observed in Fmr1KO mice.

3.5 | Molecular changes in somatosensory pain-related areas of nerve-injured WT and Fmr1KO mice

RT-PCR analysis was used to evaluate gene expression levels of pain-related proteins in the spinal cord dorsal horn, mPFC, right and left amygdalae of WT and Fmr1KO mice 21 days after nerve injury.

3.5.1 | N-acylethanolamines signalling pathway

PPARs are activated by ligands of different chemical structures, including the endocannabinoid-like compounds PEA and OEA (O'Sullivan, 2016). Considering the increased levels of PEA and OEA in the spinal cord of nerve-injured Fmr1KO mice, we evaluated *Ppara* (PPARα encoding gene) expression in spinal cord and brain areas involved in pain and emotional processing.

WT and Fmr1KO animals did not reveal significant alterations of *Ppara* expression in the spinal cord dorsal horn, though a trend to enhance *Ppara* levels was observed after nerve injury in Fmr1KO mice (Figure 5a). However, nerve-injured Fmr1KO mice showed enhanced *Ppara* expression in the mPFC when compared to sham littermates or nerve-injured WT mice (Figure 5b). In the right amygdala, a global decrease of *Ppara* expression was revealed in Fmr1KO animals compared to WT mice (Figure 5c).

Additionally, PPARα exerts anti-inflammatory effects by modulating the activity of several pro-inflammatory transcription factors, including the nuclear factor-Kappa B (NF-κB) (Rakhshandehroo et al., 2010). NF-κB promotes the expression of genes that participate in the sensitisation processes by encoding proinflammatory mediators, such as cytokines and brain-derived neurotrophic factor (Grace et al., 2014). The expression of *Rela* (NF-κB encoding gene) was not modified in the spinal cord dorsal horn and right amygdala of WT and Fmr1KO mice regardless of the surgery (Figure 5a,c). In contrast, nerve-injured Fmr1KO animals revealed increased levels of *Rela* expression in the mPFC in

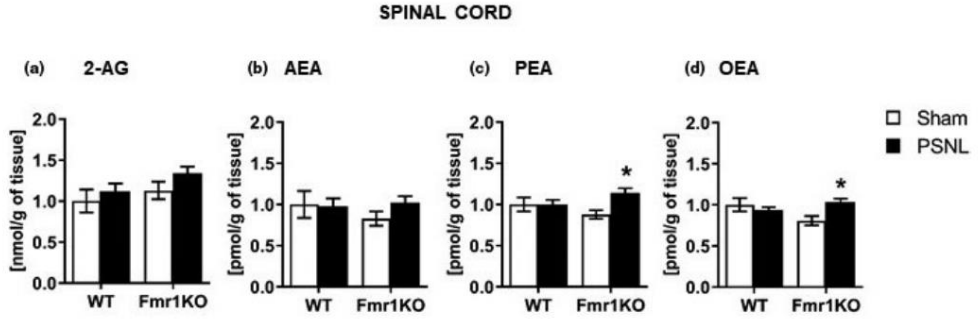

FIGURE 4 Quantification of spinal endocannabinoid levels of WT and Fmr1KO mice 21 days after sham or PSNL surgery. High-performance liquid chromatography was used to assess the concentration of 2-AG (nmol/g) (a), AEA (pmol/g) (b), PEA (pmol/g) (c) and OEA (pmol/g) (d) in the spinal dorsal horn ipsilateral to the site of injury of WT and Fmr1KO mice after a sham of PSNL surgery. Nerve-injured Fmr1KO mice showed up-regulated spinal levels of PEA and OEA compared to sham mutants, while 2-AG and AEA spinal levels were no altered. Data are expressed as mean ± *SEM* ($n = 8–11$ per group). *$p < .05$ versus Fmr1KO sham mice (ANOVA, Bonferroni test). Detailed statistical analysis is presented in Supplementary Table S3

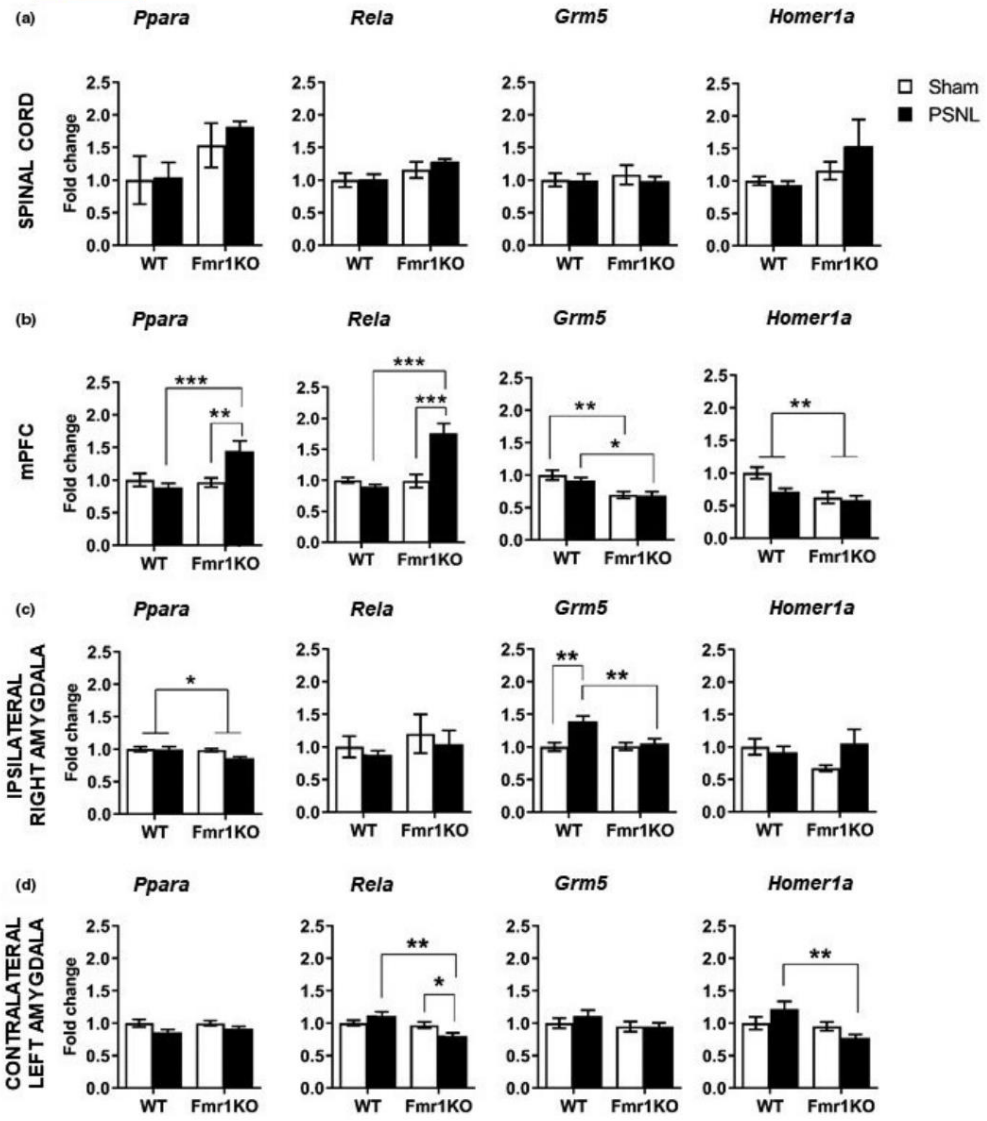

FIGURE 5 Molecular changes in somatosensory pain-related areas of WT and Fmr1KO mice 21 days after sham or PSNL surgery. RT-PCR analysis of *Ppara, Rela, Grm5 and Homer1a* gene expression in the spinal ipsilateral dorsal horn, mPFC, right and left amygdala were measured by the ΔΔCt method. Expression of all these targets was first standardized by expression of B2 M and expressed relative to the data taken from the WT sham control group. (a) In the spinal cord, RT-PCR analysis of mRNA *Ppara, Rela, Grm5 and Homer1a* expression revealed no significant differences amongst genotypes. (b) In the mPFC, nerve-injured Fmr1KO mice showed up-regulated levels of *Ppara* and *Rela* expression compared to WT PSNL and Fmr1KO sham mice, while *Grm5* expression was reduced in mutants. (c) In the right amygdala, WT mice with neuropathy exhibited increased levels of *Grm5* expression in comparison to sham littermates and nerve-injured Fmr1KO mice. (d) In the left amygdala, Fmr1KO mice after PSNL surgery showed decreased expression of *Rela* and *Homer1a* compared to nerve-injured WT animals. Data are expressed as mean ± *SEM* ($n = 6$–10 per group). *$p < .05$, **$p < .01$, ***$p < .001$ (ANOVA, Bonferroni test; Kruskal–Wallis, U Mann Whitney). Detailed statistical analysis is presented in Supplementary Table S4

comparison to sham littermates and nerve-injury WT mice (Figure 5b), whereas this gene expression was diminished in the left amygdala of Fmr1KO after nerve injury (Figure 5d).

3.5.2 | Glutamate signalling

An altered glutamate transmission mainly due to modifications in the metabotropic glutamate receptor 5 (mGluR5) has been implicated in the pathophysiological processes leading to chronic pain and associated affective states (Chung et al., 2017). mGluR5 forms a complex with the Homer Scaffolding Protein 1a (HOMER1a) that plays an important role in glutamate-mediated cellular signalling and nociception (Obara, Goulding, Hu, et al., 2013). We have evaluated the expression levels of the gene coding for mGluR5 protein (*Grm5*) and the HOMER1A encoding gene (*Homer1a*). *Grm5* gene expression was not altered in the spinal cord and left amygdala of WT and mutant mice with or without nerve ligation (Figure 5a,d). However, sham and nerve-injured Fmr1KO mice showed down-regulated mRNA levels of *Grm5* in the mPFC compared to the respective WT groups (Figure 5b). Moreover, WT mice showed increased levels of *Grm5* expression after nerve injury in the right amygdala when compared to sham littermates and nerve-injured Fmr1KO mice (Figure 5c,d). The expression of *Homer1a* was not modified in the spinal cord dorsal horn nor right amygdala of WT and Fmr1KO mice regardless of the surgery (Figure 5a,c). In the mPFC, a global decrease of *Homer1a* expression was revealed in Fmr1KO animals in comparison to WT mice (Figure 5b). However, *Homer1a* expression was decreased in the left amygdala after PSNL surgery in Fmr1KO mice compared to nerve-injured WT animals (Figure 5d).

3.6 | Correlations between gene expression and the time of immobility in the water maze

In order to provide additional findings in support of the role of the different brain areas on the effect of Fmr1 gene promoting depressive-like behaviour after nerve injury, we evaluated the correlations between gene expression and immobility time of WT and Fmr1KO mice regardless of the type of surgery. Elevated time of immobility in the forced swim test maze was positively correlated with the expression in the left amygdala (contralateral to the site of injury) of Rela ($r = 0.567$; $p = .007$), Grm5 ($r = 0.507$; $p = .016$) and Homer1a ($r = 0.596$; $p = .003$), but not with Ppara expression ($r = 0.007$; $p = .976$) (Figure 6a-d). These correlations and the observed decreased gene expression of Homer1a and Rela in the left amygdala of nerve-injured Fmr1KO mice (Figure 5d) suggest a possible role of the left amygdala in the protective phenotype of Fmr1KO mice against the nociceptive and emotional manifestations of neuropathic pain. No association was revealed between time of immobility and mPFC expression of Ppara ($r = -0.067$; $p = .761$), Rela ($r = 0.256$; $p = .227$), Grm5 ($r = 0.163$; $p = .406$) and Homera1 ($r = -0.041$; $p = .834$). Similarly, no significant associations were revealed in the right amygdala for Ppara ($r = 0.088$; $p = .697$), Rela ($r = 0.162$; $p = .471$), Grm5 ($r = 0.357$; $p = .103$) and Homera1 ($r = -0.280$; $p = .207$) expression (data not shown).

4 | DISCUSSION

This study provides novel findings to clarify the mechanisms involved in the resistant phenotype of Fmr1KO mice, a mouse model of Fragile X syndrome, against the nociceptive,

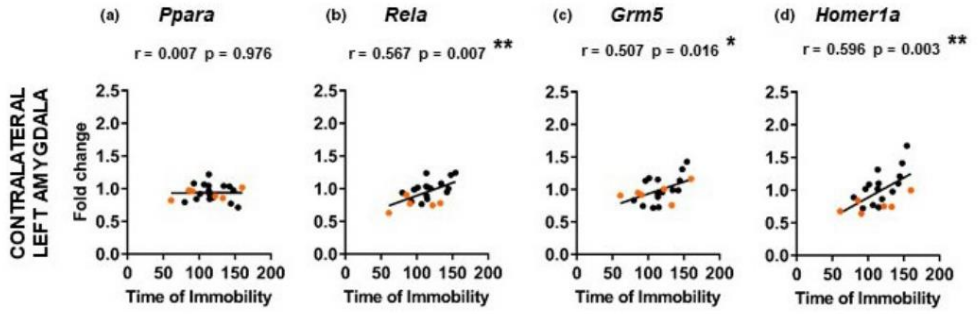

FIGURE 6 Correlations between gene expression in the left amygdala and depressive-like behaviour of WT and Fmr1KO mice 21 days after sham or PSNL surgery. Correlation between the time of immobility in the forced swimming test and the gene expression of *Ppara* (a), *Rela* (b), *Grm5* (c) *and Homer1a* (d) in the left amygdala of WT and Fmr1KO mice. *Rela, Grm5 and Homer1a* gene expression in the left amygdala, but no *Ppara*, showed significant positive correlations with depressive-like behaviours. Orange dots represent PSNL Fmr1KO mice. Data are expressed as mean ($n = 5$–7 per group). $^{*}p < .05$, $^{**}p < .01$ (Pearson Correlation)

cognitive and affective manifestations of neuropathic pain. We reveal the participation of CB2 on the protective effects of this phenotype in the development of neuropathic pain and identify specific changes in the endocannabinoid system and the expression of pain-related genes in spinal and supraspinal areas in this mouse model protected against chronic pain manifestations.

Nerve injury induced the expected mechanical allodynia and heat hypersensitivity in WT mice (La Porta et al., 2016), whereas both nociceptive manifestations were attenuated in Fmr1KO mice during at least 2 weeks after nerve ligation. In agreement, previous studies have reported that Fmr1KO mice failed to show enhanced mechanical and thermal hypersensitivity in response to nerve injury (Price et al., 2007; Wang et al., 2016). We investigated for the first time the emotional and cognitive manifestations of neuropathic pain in Fmr1KO mice. Neuropathic pain impaired long-term memory and produced depressive-like behaviour in WT mice, as previously reported (Chung et al., 2017; Martínez-Navarro et al., 2019). The lack of the *Fmr1* gene has been widely related to memory deficits in mice (Gomis-González et al., 2016). Accordingly, Fmr1KO mice showed cognitive impairment in sham and neuropathic pain conditions. Furthermore, Fmr1KO mice did not develop depressive-like behaviour associated with nerve injury, suggesting the participation of FRMP in this emotional manifestation of chronic pain.

Fmr1KO mice with a partial deletion of CB2 and their WT littermates were used to evaluate the involvement of this receptor in the neuropathic pain-resistant phenotype displayed by Fmr1KO mice. CB2 presence was required to obtain the protective phenotype on nociceptive and emotional responses, since the WT phenotype induced by PSNL was rescued when CB2 was partially removed in Fmr1KO mice. In addition, sham mice partially lacking CB2 showed an enhanced depressive-like behaviour, suggesting an antidepressant function of CB2 under basal conditions. Accordingly, previous studies targeting CB2 with pharmacological tools or genetic deletion have also shown antidepressant effects of CB2 activation (Ishiguro et al., 2018; Liu et al., 2017). Moreover, sham mice partially lacking CB2 did not show cognitive deficits under basal conditions, as other studies have reported when this receptor was pharmacologically blocked (Busquets-Garcia et al., 2013). Similarly, CB2 deletion did not modify the cognitive impairment associated with the nerve injury or with the lack of FMRP, ruling out a function of CB2 promoting cognitive impairment. Altogether, these results highlight that CB2 participates in the protective phenotype displayed by Fmr1KO mice against the nociceptive and emotional manifestations of neuropathic pain and prevents depressive-like behaviour in WT mice under basal conditions.

Sciatic nerve injury increased PEA and OEA levels in the spinal cord of Fmr1KO mice, but not in WT mice. This enhanced tone of spinal N-acylethanolamines could participate in the attenuation of nociceptive responses observed in Fmr1KO mice after the nerve injury. In agreement, previous studies have shown that increased levels of PEA and OEA alleviate different chronic pain states (Gugliandolo et al., 2018; Suardíaz et al., 2007). In contrast, neuropathic pain was not associated in our study with altered levels of 2-AG or AEA at the spinal level, neither in WT or Fmr1KO mice. Previous studies showed that changes in 2-AG and AEA at the spinal level were dependent on time at early periods after nerve injury (from day 3 to 7) (Petrosino et al., 2007), whereas no major changes in endocannabinoids were revealed by other authors at early stages (Starowicz et al., 2013; Thomas et al., 2020). However, previous studies did not investigate the changes in endocannabinoid levels at late periods, such as in our study (21 days after nerve injury).

Taken into account these changes in N-acylethanolamines, we investigated the expression of specific genes related to ethanolamine pathways and pain processing including *Ppara*, *Rela*, *Grm5* and *Homer1a*. PPARα, the protein encoded by *Ppara*, is mainly activated by PEA and OEA and is involved in nociception modulating the activity of pro-inflammatory factors such as NF-κB, encoded by *Rela* (Di Marzo, 2018; Iannotti et al., 2016). Nerve ligation did not modify *Ppara* and *Rela* gene expression in the spinal dorsal horn of WT or Fmr1KO mice. In accordance, previous studies did not reveal alterations in *Ppara* expression in the spinal cord of mice after peripheral nerve injury (Okine et al., 2015). We presume that the absence of alterations in *Rela* expression in our experimental conditions could be explained by the absence of changes in *Ppara* gene expression (Rakhshandehroo et al., 2010).

The mGluR5–Homer1a complex, formed by the proteins encoded by these genes, is implicated in the development of chronic pain and associated negative affective states through glutamate-mediated cellular signalling (Chung et al., 2017; Obara, Goulding, Gould, et al., 2013). The expression levels of *Grm5* and *Homer1a* in the spinal dorsal horn of WT or Fmr1KO mice also remained unaltered after nerve injury. Accordingly, no alterations of mGluR5 or Homer1a expression were previously reported following peripheral nerve injury (Michot et al., 2017; Obara, Goulding, Hu, et al., 2013). This absence of expression changes highlights the complex nature of pain processing and prompted us to search for molecular mechanisms in other somatosensory areas that could explain the protective phenotype of Fmr1KO mice.

The expression of *Ppara* and *Rela* in the mPFC was enhanced after nerve injury in Fmr1KO, but not in WT mice. This cortical *Ppara* enhancement may potentially prevent the nociceptive manifestations of neuropathic pain in Fmr1KO mice considering the antinociceptive effects reported by PPARα activation (Di Marzo, 2018). Though PPARα induces antinociceptive effects by preventing the upregulation of the protein product of *Rela* (NF-κB)

(Iannotti et al., 2016), a previous study demonstrated that PPARα agonist administration did not modify mRNA levels of *Rela* (Nakano et al., 2018). Therefore, *Rela* modifications in Fmr1KO mice could be independent of *Ppara*. Moreover, Fmr1KO mice exhibited diminished *Grm5* and *Homer1a* expression in the mPFC under sham and neuropathic pain conditions. The complex mGluR5–Homer1a increases glutamatergic input and nociceptive transmission under neuropathic pain conditions (Obara, Goulding, Hu, et al., 2013) and the blockade of cortical mGluR5 decreases mechanical and thermal hypersensitivity after spinal nerve injury (Chung et al., 2017). Hence, the reduced cortical expression of the genes encoding for the pronociceptive mGluR5–Homer1a complex may also contribute to the attenuated nociceptive manifestations of Fmr1KO mice after nerve ligation.

Nerve-injured WT mice showed increased Grm5 expression restricted to the right amygdala, in agreement with previous studies describing the enhanced activity of the right amygdala in chronic pain conditions (Allen et al., 2020). Indeed, previous reports described increased mGlur5 function in the right amygdala of mice subjected to different pain models (Crock et al., 2012; Kolber et al., 2010). Interestingly, Fmr1KO mice did not show this increase in Gmr5 expression and exhibited decreased Ppara expression in the same area. Previous studies showed that mGluR5 inhibition in this brain area decreased nociceptive responses in different chronic pain models (Crock et al., 2012; Kolber et al., 2010), suggesting that the unaltered expression of Grm5 in the right amygdala of Fmr1KO mice could participate in the antinociceptive phenotype observed in these animals. Unexpectedly, nerve ligation induced also significant changes in the left amygdala of Fmr1KO mice, involving significant down-regulations of *Rela* and *Homer1a*. Positive correlations between the expression of the pronociceptive genes *Rela* and *Homer1a* (Obara, Goulding, Hu, et al., 2013; Paterniti et al., 2017) and the time of immobility in the forced swimming test were obtained exclusively in the contralateral left amygdala of WT and Fmr1KO mice. In agreement, preclinical animal models have demonstrated divergent functions of the left and right amygdala (Cooper et al., 2018; Sadler et al., 2017). Thus, our data are compatible with participation of the left amygdala in the depressive-like phenotype associated with chronic pain conditions, suggesting that the decreased expression of *Rela* and *Homer1a* in the contralateral left amygdala could prevent this emotional manifestation of chronic pain.

Overall, we show that CB2 is required to obtain a protective phenotype against the nociceptive and emotional manifestations of neuropathic pain in Fmr1KO mice. Increased spinal levels of PEA and OEA and decreased expression of glutamate-transmission genes at supra-spinal levels are associated with the attenuated nociceptive responses of Fmr1KO mice. In addition, our findings support the lateralized activity of the left and right amygdala to modulate the affective dimension of chronic pain and highlight the role of glutamate transmission-related genes in this area in the prevention of depressive-like behaviour associated with chronic pain conditions. The present data underline the interest of CB2 as a target to treat neuropathic pain since its low presence in neuronal cells (Shang & Tang, 2017) could avoid the psychoactive side effects of cannabinoid drugs (Davis, 2014). Nevertheless, it would be important in future investigations to include multidisciplinary techniques, such as high-resolution brain imaging or electrophysiological tools, which represent experimental approaches more closely related to human pain experience to potentially optimize preclinical drug discovery aimed at alleviating neuropathic pain.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS CONTRIBUTIONS

R.M. and A.O. designed the study and obtained grants to fund it. C.LP. performed preliminary behavioural experiments. A.R-L. performed the behavioural and molecular experiments and statistical analysis. D.C. contributed to data analysis. A.P. and R.T. carried out and provided resources for the HPLC analysis. A.R-L. wrote the manuscript and R.M. and D.C. reviewed and revised the manuscript and contributed to the interpretation of findings. All authors have read and approved the final manuscript. All authors discussed the results, revised the manuscript critically for important content and approved the final version All authors discussed the results, revised the manuscript critically for important content and approved the final version.

DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the paper and its Supporting Information files or from the authors upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Article #2

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eLife RESEARCH ARTICLE

Protective role of neuronal and lymphoid cannabinoid CB_2 receptors in neuropathic pain

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Abstract Cannabinoid CB_2 receptor (CB_2) agonists are potential analgesics void of psychotropic effects. Peripheral immune cells, neurons and glia express CB_2; however, the involvement of CB_2 from these cells in neuropathic pain remains unresolved. We explored spontaneous neuropathic pain through on-demand self-administration of the selective CB_2 agonist JWH133 in wild-type and knockout mice lacking CB_2 in neurons, monocytes or constitutively. Operant self-administration reflected drug-taking to alleviate spontaneous pain, nociceptive and affective manifestations. While constitutive deletion of CB_2 disrupted JWH133-taking behavior, this behavior was not modified in monocyte-specific CB_2 knockouts and was increased in mice defective in neuronal CB_2 knockouts suggestive of increased spontaneous pain. Interestingly, CB_2-positive lymphocytes infiltrated the injured nerve and possible CB_2transfer from immune cells to neurons was found. Lymphocyte CB_2depletion also exacerbated JWH133 self-administration and inhibited antinociception. This work identifies a simultaneous activity of neuronal and lymphoid CB_2that protects against spontaneous and evoked neuropathic pain.

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Introduction

Cannabinoid CB_2 receptor (CB_2) agonists show efficacy in animal models of chronic inflammatory and neuropathic pain, suggesting that they may be effective inhibitors of persistent pain in humans (***Bie et al., 2018***; ***Maldonado et al., 2016***; ***Shang and Tang, 2017***). However, many preclinical studies assess reflexive-defensive reactions to evoked nociceptive stimuli and fail to take into account spontaneous pain, one of the most prevalent symptoms of chronic pain conditions in humans (***Backonja and Stacey, 2004***; ***Mogil et al., 2010***; ***Rice et al., 2018***) that triggers coping responses such as analgesic consumption. As a consequence, conclusions drawn from animal models relying on

evoked nociception may not translate into efficient pharmacotherapy in humans (**Huang et al., 2019**; **Mogil, 2009**; **Percie du Sert and Rice, 2014**), which underlines the need to apply more sophisticated animal models with clear translational value. Operant paradigms in which animals voluntarily self-administer analgesic compounds can provide high translatability and also identify in the same experimental approach potential addictive properties of the drugs (**Mogil, 2009**; **Mogil et al., 2010**; **O'Connor et al., 2011**). In this line, a previous work using a CB_2 agonist, AM1241, showed drug-taking behavior in nerve-injured rats and not in sham-operated animals, suggesting spontaneous pain relief and lack of abuse potential of CB_2agonists (**Gutierrez et al., 2011**), although the possible cell populations and mechanisms involved remain unknown. In addition, a recent multicenter study demonstrated off-target effects of this compound on anandamide reuptake, calcium channels and serotonin, histamine and kappa opioid receptors (**Soethoudt et al., 2017**).

CB_2, the main cannabinoid receptors in peripheral immune cells (**Fernández-Ruiz et al., 2007**; **Schmöle et al., 2015a**), are found in monocytes, macrophages and lymphocytes, and their expression increases in conditions of active inflammation (**Schmöle et al., 2015b**; **Shang and Tang, 2017**). The presence of CB_2 in the nervous system was thought to be restricted to microglia and limited to pathological conditions or intense neuronal activity (**Manzanares et al., 2018**). However, recent studies using electrophysiological approaches and tissue-specific genetic deletion revealed functional CB_2 also in neurons, where they modulate dopamine-related behaviors (**Zhang et al., 2014**) and basic neurotransmission (**Quraishi and Paladini, 2016**; **Stempel et al., 2016**). Remarkably, the specific contribution of immune and neuronal CB_2 to the development of chronic pathological pain has not yet been established.

This work investigates the participation of neuronal and non-neuronal cell populations expressing CB_2 in the development and control of chronic neuropathic pain. We used a pharmacogenetic strategy combining tissue-specific CB_2 deletion and drug self-administration to investigate spontaneous neuropathic pain. Constitutive and conditional knockouts lacking CB_2 in neurons or monocytes were nerve-injured, subjected to operant self-administration of the specific CB_2 agonist JWH133 (**Soethoudt et al., 2017**) and were evaluated for nociceptive and anxiety-like behavior. We also explored infiltration of CB_2-positive immune cells in the injured nerve of mice receiving bone marrow transplants from CB_2-GFP BAC mice. Finally, immunological blockade of lymphocyte extravasation was used to investigate the effect of this cell type on spontaneous neuropathic pain and its involvement on the pain-relieving effects of the cannabinoid CB_2 agonist.

Results

Self-administration of a CB_2 receptor agonist to alleviate spontaneous pain and anxiety-associated behavior

CB_2 agonists have shown efficacy reducing evoked sensitivity and responses of negative affect in mouse models of chronic pain (**Maldonado et al., 2016**). Although antinociception is a desirable characteristic for drugs targeting chronic neuropathic pain, it is unclear whether the pain-relieving effects of the CB_2 agonist would be sufficient to elicit drug-taking behavior in mice and the cell populations involved. To answer these questions, mice underwent a PSNL or a sham surgery and were placed in operant chambers where they had to nose poke on an active sensor to obtain i.v. self-administration of the CB_2 agonist JWH133 or vehicle (**Figure 1A**). Sham mice or nerve-injured animals receiving vehicle or the low dose of JWH133 (0.15 mg/kg/inf) did not show significant differences in active nose-poking during the last 3 days of the drug self-administration period (**Figure 1B**, **Figure 1—figure supplement 1A**). Conversely, nerve-injured mice exposed to the high dose of JWH133 (0.3 mg/kg/inf) showed higher active responses than sham-operated mice receiving the same treatment (**Figure 1B**, **Figure 1—figure supplement 1A**). As expected, the operant behavior of sham-operated mice exposed to JWH133 was not different from that of sham mice exposed to vehicle, suggesting absence of reinforcing effects of the CB_2 agonist in mice without pain (**Figure 1B**, **Figure 1—figure supplement 1A**). The number of nose pokes on the inactive sensor was similar among the groups, indicating absence of locomotor effects of the surgery or the pharmacological treatments. Thus, operant JWH133 self-administration was selectively associated to the neuropathic condition.

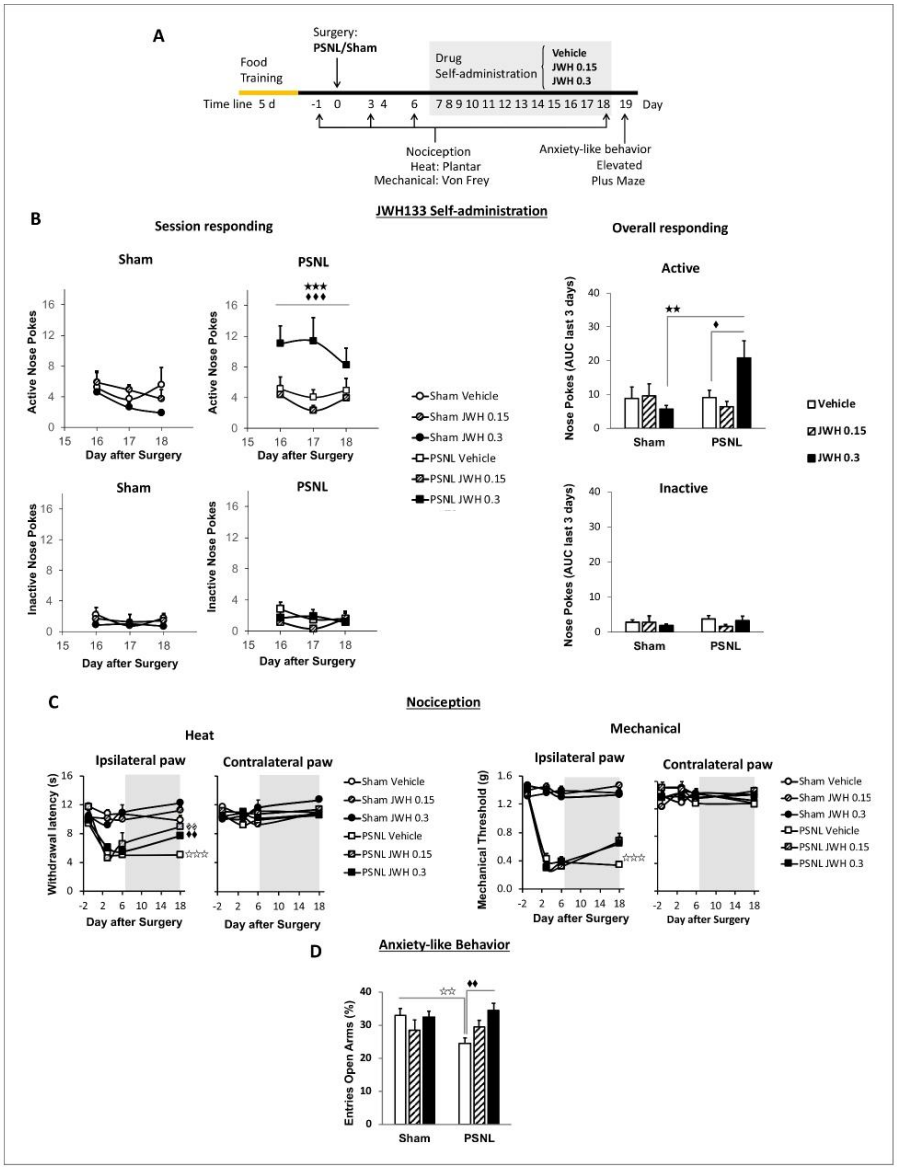

Figure 1. C57BL/6J mice self-administer a CB_2 receptor agonist with antinociceptive and anxiolytic-like properties. (**A**) Timeline of the drug self-administration paradigm. Mice were trained in Skinner boxes (5 days, 5d) where nose-poking an active sensor elicited delivery of food pellets. Partial sciatic nerve ligation (PSNL) or sham surgery were conducted (day 0) followed by jugular catheterization to allow intravenous (i.v.) drug infusion. From days 7 to 18, mice returned to the operant chambers and food was substituted by i.v. infusions of JWH133 (0.15 or 0.3 mg/kg/inf.). Mechanical and

Figure 1 continued on next page

Figure 1 continued

thermal sensitivity were assessed before (−1) and 3, 6 and 18 days after PSNL using Plantar and von Frey tests. Anxiety-like behavior was measured at the end (day 19) with the elevated plus maze. (**B**) Nerve-injured mice poked the active sensor to consume the high dose of JWH133 (0.3 mg/kg/inf.). (**C**) PSNL-induced ipsilateral thermal and mechanical sensitization (days 3 and 6). JWH133 inhibited thermal hypersensitivity but the effect on mechanical nociception was not significant (**D**) Nerve-injured mice receiving vehicle showed decreased percentage of entries to the open arms of the elevated plus maze, whereas PSNL mice receiving JWH133 0.3 mg/kg/inf. did not show this alteration. N = 5–10 mice per group. Shaded areas represent drug self-administration. Mean and error bars representing SEM are shown. Stars represent comparisons vs. sham; diamonds vs. vehicle. *p<0.05; **p<0.01; ***p<0.001.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. JWH133 self-administration, antinociception and anxiolytic-like effects in C57BL6/J mice.

Figure supplement 1. JWH133 self-administration after nerve injury or sham surgery in C57BL6/J mice and food-maintained operant training before the drug self-administration.

Figure supplement 1—source data 1. Operant training and full JWH133 self-administration in C57BL6/J mice.

Nociceptive responses to thermal and mechanical stimuli were assessed before and after the self-administration period (days −1, 3, 6 and 18). Before the treatment with the CB_2 agonist, all nerve-injured mice developed heat and mechanical hypersensitivity in the ipsilateral paw (***Figure 1C***). After self-administration (shaded area, ***Figure 1C***) mice exposed to JWH133 showed a significant reduction in heat hypersensitivity (***Figure 1C***, day 18, ipsilateral paw), although the alleviation of mechanical hypersensitivity did not reach statistical significance in this experiment. No significant drug effects were observed in the contralateral paws.

We also studied affective-like behavior in mice exposed to this chronic pain condition. Anxiety-like behavior was enhanced in nerve-injured mice treated with vehicle, as these mice visited less frequently the open arms of the elevated plus maze (***Figure 1D***). This emotional response was absent in nerve-injured mice repeatedly exposed to the high dose of JWH133 (***Figure 1D***). Therefore, the high dose of JWH133 elicited a drug-taking behavior selectively associated to spontaneous pain relief, and had efficacy limiting the pronociceptive effects of the nerve injury and its emotional-like consequences.

CB_2 receptor mediates JWH133 effects on spontaneous pain alleviation

JWH133 has been recently recommended as a selective CB_2 agonist to study the role of CB_2 in biological and disease processes due to its high selectivity for this receptor (***Soethoudt et al., 2017***). To investigate the specificity of the CB_2 agonist in our model, the high dose of JWH133 (0.3 mg/kg/inf) was offered to nerve-injured mice constitutively lacking the CB_2 (CB_2 KO) and to C57BL/6J wild-type mice. CB_2 KO mice showed a significant disruption of JWH133-taking behavior on the last sessions of the drug self-administration period (***Figure 2A***, ***Figure 2—figure supplement 1A***). Overall discrimination between the active and inactive sensors was also significantly blunted in CB_2 KO mice (Source Data File) and inactive nose pokes were similar in both groups of mice, indicating absence of genotype effect on locomotion (***Figure 2A***, ***Figure 2—figure supplement 1A***). The disruption of drug-taking behavior shown in CB_2 KO mice was accompanied by an inhibition of JWH133 effects on nociceptive and affective behavior (***Figure 2B***, ***Figure 2C***).

CB_2 KO and C57BL/6J mice developed similar thermal and mechanical hypersensitivity in the injured paw (***Figure 2B***, day 6, Ipsilateral paw), although CB_2 KO mice also developed hypersensitivity in the contralateral paw, as previously described (***Racz et al., 2008***). While C57BL/6J mice showed significant recovery of thermal and mechanical thresholds after JWH133 self-administration (***Figure 2B***, day 18), CB_2 KO mice showed no effects of the treatment on mechanical sensitivity (***Figure 2B***, day 18, Mechanical) and a partial recovery of the thresholds to heat stimulation (***Figure 2B***, day 18, Heat). Contralateral mechanical sensitization was still present in CB_2 KO mice exposed to the CB_2 agonist (***Figure 2B***, Contralateral paw). Likewise, nerve-injured C57BL/6J mice showed less anxiety-like behavior after JWH133 self-administration than CB_2 KO mice (***Figure 2C***), suggesting that these anxiolytic-like effects of JWH133 are mediated by CB_2. Hence, CB_2 KO mice showed reduced drug-taking behavior accompanied by blunted inhibition of JWH133 effects on mechanical nociception and anxiety-like behavior, confirming mediation of these effects by CB_2.

JWH133 has shown effects interacting with the Transient Receptor Potential Ankyrin1 (TRPA1) (***Soethoudt et al., 2017***), a receptor needed for thermal pain perception (***Vandewauw et al.,***

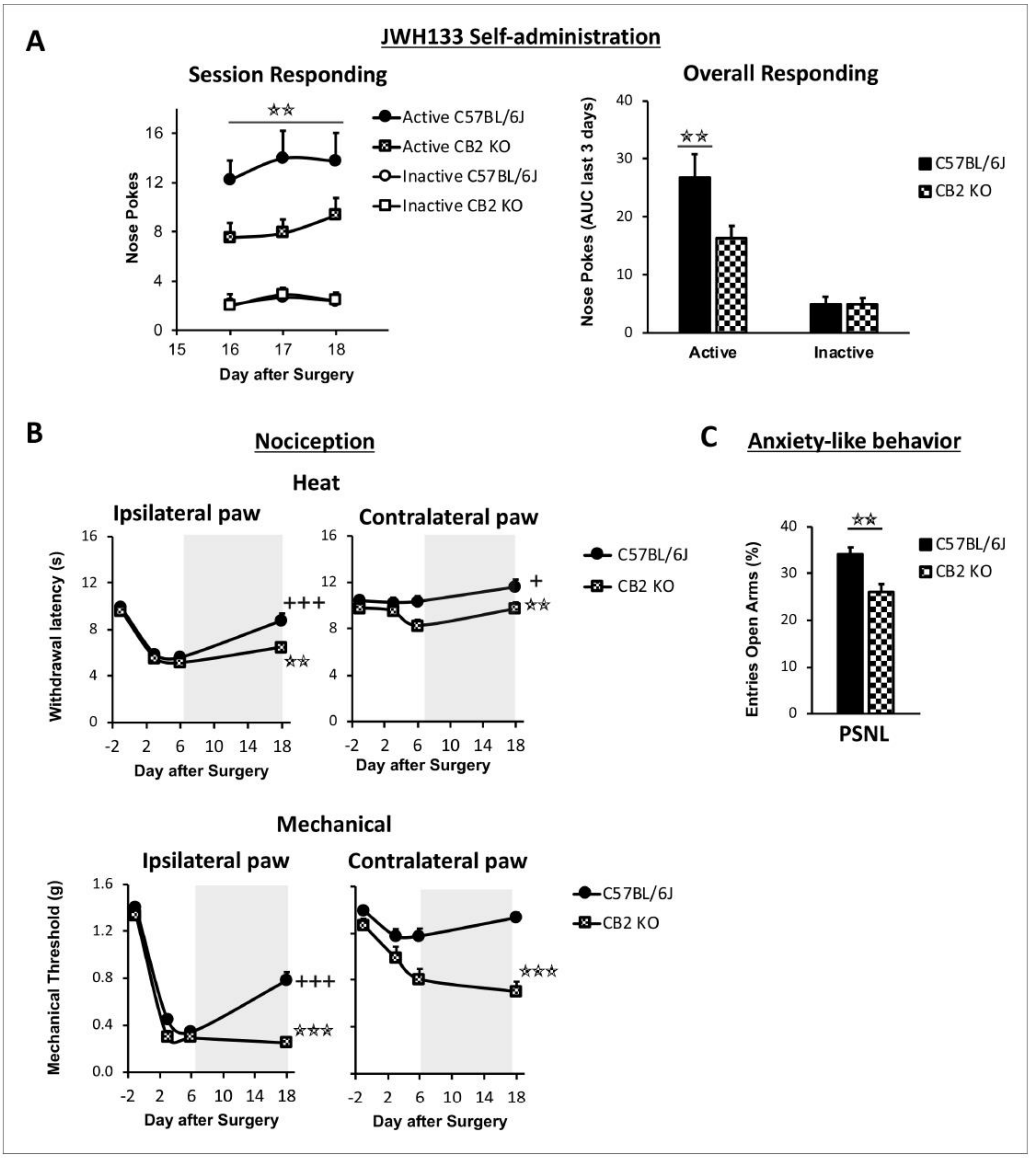

Figure 2. Nerve-injured mice constitutively lacking CB_2 receptor show disruption of JWH133 intake and blunted effects of the drug. CB_2 constitutive knockout mice (CB_2 KO) and C57BL/6J mice were food-trained in Skinner boxes (Food training, 5 days), subjected to a partial sciatic nerve ligation (PSNL, day 0), catheterized and exposed to high doses of the CB_2 agonist JWH133 (0.3 mg/kg/inf., days 7 to 18). Nociceptive sensitivity to heat (Plantar) and mechanical (von Frey) stimulation were measured before and after the nerve injury (−1,3,6,18), and anxiety-like behavior was evaluated at the end

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(day 19). (**A**) CB_2 KO mice showed decreased active operant responding for the CB_2 agonist. (**B**) The effects of JWH133 on thermal nociception were reduced in constitutive knockout mice. CB_2 KO mice showed contralateral mechanical and thermal sensitization and complete abolition of JWH133 effects on mechanical hypersensitivity. (**C**) Anxiety-like behavior after the treatment worsened in CB_2 KO mice. N = 16–19 mice per group. Mean and error bars representing SEM are shown. Shaded areas represent drug self-administration. Stars represent comparisons vs. C57BL/6J mice; crosses represent day effect. *p<0.05; **p<0.01; ***p<0.001.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. JWH133 self-administration, antinociception and anxiolytic-like effects in nerve-injured CB_2 constitutive knockout mice.
Figure supplement 1. JWH133 self-administration in C57BL6/J and CB_2 constitutive knockout (CB2 KO) mice and food-maintained operant training before nerve injury and drug self-administration.
Figure supplement 1—source data 1. Operant training and full JWH133 self-administration in CB_2 constitutive knockout mice.
Figure supplement 2. Mice lacking TRPA1 receptor retain JWH133- effects.
Figure supplement 2—source data 1. Antinociceptive effect of JWH133 in TRPA1 knockout mice.

2018), that could also participate in other nociceptive responses. In order to assess a possible effect of the CB2 agonist on TRPA1 receptors in vivo, we conducted an additional experiment in which we compared the antinociceptive efficacy of JWH133 in sham and nerve-injured TRPA1 knockout mice (TRPA1 KO) and wild-type mice. After 7 days of the nerve injury, vehicle or i.p. doses of JWH133 (5 and 10 mg/kg) were administered to nerve-injured and sham-operated mice, and mechanical and heat nociception were assessed 30 and 75 min later, respectively. We observed similar effects of JWH133 inhibiting mechanical hypersensitivity in TRPA1 KO and WT mice (***Figure 2—figure supplement 2A***). Interestingly, TRPA1 KO mice showed a prominent inhibition of neuropathic thermal hypersensitivity (***Figure 2—figure supplement 2B***). In spite of this lack of sensitivity, a significant general effect was observed in nerve-injured mice with the high dose of JWH133 (10 mg/kg), regardless of the genotype of the mice. Thus, the results on mechanical sensitivity suggest that these effects are not due to an interaction of the drug with the TRPA1 receptor. The lack of thermal hypersensitivity observed in the TRPA1 KO mice may occlude possible JWH133 effects on neuropathic thermal hyperalgesia through TRPA1; however, a significant effect of JWH133 was observed in both strains after the nerve injury, suggesting that at least the CB2 receptor is involved in the inhibitory effect on thermal hyperalgesia.

Participation of neuronal and monocyte CB_2 receptor in neuropathic pain symptomatology

CB_2 receptors were initially described in peripheral immune cells (***Munro et al., 1993***), although they have been found in multiple tissues including the nervous system. In order to distinguish the participation of CB_2 from different cell types on spontaneous neuropathic pain, we conducted the self-administration paradigm in nerve-injured mice lacking CB_2 in neurons (Syn-Cre+ mice) or in monocyte-derived cells (LysM-Cre+) and in their wild-type littermates (Cre Neg). Syn-Cre+ mice showed increased active operant responding for JWH133 (***Figure 3A***, ***Figure 3—figure supplement 1A***), suggesting increased spontaneous pain and possible decrease of drug effects. On the other hand, LysM-Cre+ mice did not show significant alteration of drug-taking behavior (***Figure 3A***, ***Figure 3—figure supplement 1A***). Inactive responding was also similar between Cre Neg and knockout mice. Thus, data from the drug self-administration experiments showed persistence of drug effects in the different genotypes and increased self-administration in mice lacking neuronal CB_2, suggestive of increased spontaneous pain.

We also measured antinociceptive and anxiolytic-like effects of JWH133 self-administration (***Figure 3B***, ***Figure 3C***). The three mouse lines showed similar evoked responses to nociceptive stimulation after nerve injury (***Figure 3B***). A slight but significant impairment on the effect of JWH133 on mechanical sensitivity was found in Syn-Cre+ mice (***Figure 3C***) in spite of the increased JWH133 consumption, compatible with reduced efficacy of JWH133 in this mouse strain. The assessment of anxiety-like behavior did not reveal apparent differences among the three genotypes (***Figure 3C***). Thus, the increased JWH133 consumption observed in Syn-Cre+ mice was not reflected in increased anxiolysis and JWH133 antinociceptive effects were blunted, suggesting partial involvement of neuronal CB_2 in the development of spontaneous and evoked neuropathic pain. To investigate a possible involvement of peripheral neuronal CB_2 on the antinociceptive effects of JWH133, an additional

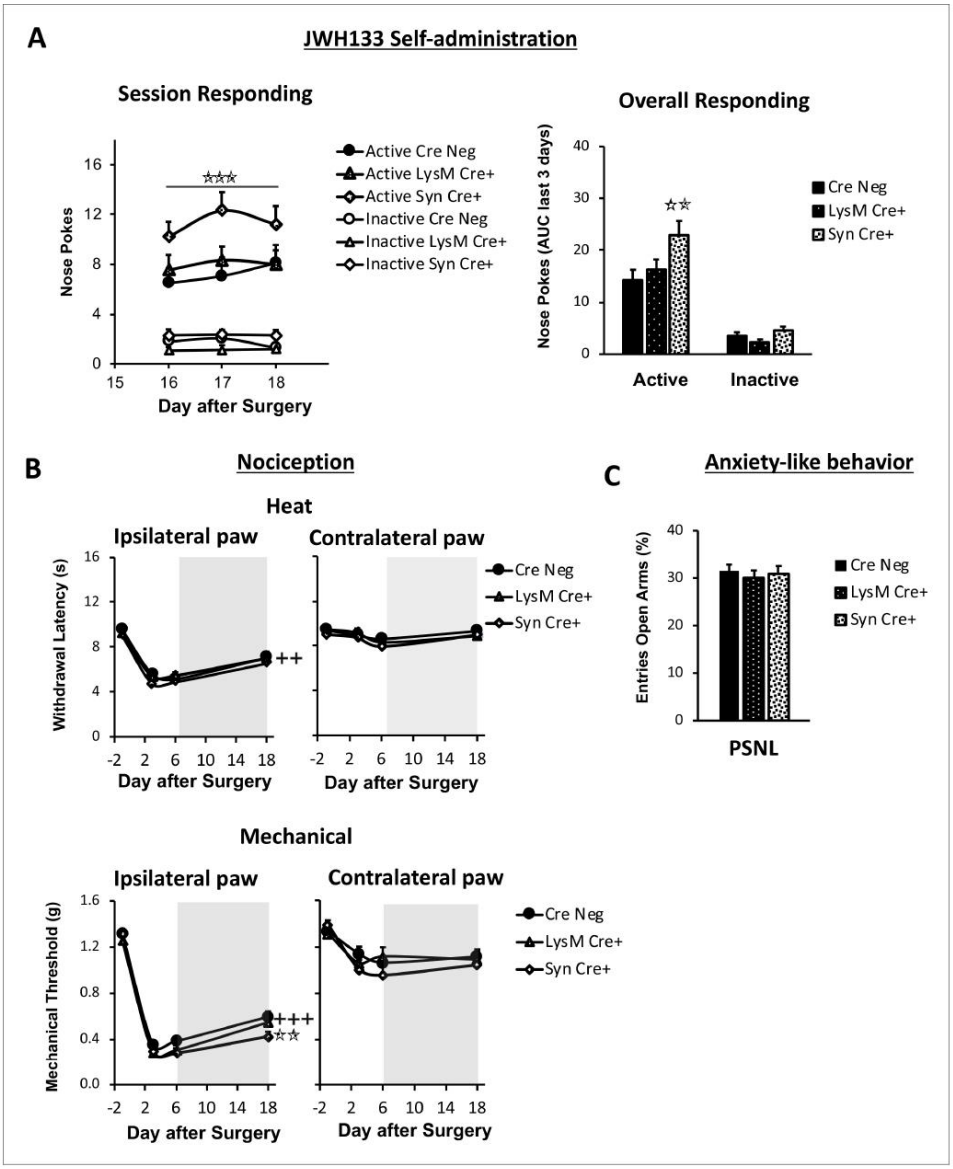

Figure 3. Nerve-injured mice defective in neuronal CB_2 receptor show increased self-administration of the CB_2 agonist JWH133 and a decrease in the antinociceptive effects of the drug. Mice lacking CB_2 in neurons (Syn-Cre+), in monocytes (LysM-Cre+) or their wild-type littermates (Cre Neg) were food-trained in Skinner boxes (Food training, 5 days), subjected to partial sciatic nerve ligation (PSNL, day 0), catheterized and exposed to JWH133 (0.3 mg/kg/inf., days 7 to 18). Nociceptive sensitivity to heat (Plantar) and mechanical (von Frey) stimulation were measured before and after nerve injury

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Figure 3 continued

(−1,3,6,18), anxiety-like behavior was evaluated at the end (day 19). (**A**) Syn-Cre+ mice showed increased active operant responding for JWH133 in the last sessions of the self-administration period (**B**) All mouse strains showed decreased heat nociception after JWH133 treatment, and Syn-Cre+ mice showed reduced effects of JWH133 on mechanical nociception. (**C**) Every mouse strain showed similar anxiety-like behavior after JWH133 self-administration. No significant differences were found between LysM-Cre+ and Cre Neg mice. N = 18–36 mice per group. Mean and error bars representing SEM are shown. Shaded areas represent drug self-administration. Stars represent comparisons vs. Cre Neg mice; crosses represent day effect. *p<0.05; **p<0.01; ***p<0.001.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. JWH133 self-administration, antinociception and anxiolytic-like effects in nerve-injured neuronal or microglial CB_2 knockout mice.
Figure supplement 1. JWH133 self-administration in mice lacking CB_2 in neurons or monocytes and their wild-type littermates and food-maintained operant training before nerve injury and drug self-administration.
Figure supplement 1—source data 1. Operant training and full JWH133 self-administration in neuronal or microglial CB_2 knockout mice.
Figure supplement 2. Mice lacking CB_2 in Nav1.8+ peripheral neurons show unaltered JWH133 antinociceptive effects.
Figure supplement 2—source data 1. Antinociceptive effect of JWH133 in CB_2 Nav1.8 Cre+ mice lacking CB2 in primary afferent neurons.

experiment was performed in floxed CB_2 mice expressing Cre recombinase in Nav1.8+ primary afferents (Nav1.8-Cre+, lacking CB_2 only in primary afferent nociceptive fibers, ***Figure 3—figure supplement 2***), and in floxed littermates lacking Cre (Cre Neg). After 7 days of the nerve injury, vehicle or i.p. doses of JWH133 (5 and 10 mg/kg) were administered to nerve-injured and sham-operated mice, and mechanical and heat nociception were assessed 30 and 75 min later, respectively. No significant differences were observed between both genotypes in mechanical or thermal sensitivity (***Figure 3—figure supplement 2***) revealing that CB_2 primarily expressed in nociceptors were not involved in the antinociceptive effects of JWH133.

Infiltration of non-neuronal CB_2 receptor-GFP+ cells in the injured nerve

The persistence of JWH133 effects after genetic deletion of CB_2 from neurons and monocyte-derived cells led us to hypothesize that CB_2 of other cell types may still exert neuromodulatory effects. To investigate possible infiltration of non-neuronal GFP+ cells in the injured nerve, we transplanted bone marrow cells from C57BL/6J or CB_2-GFP BAC mice to lethally irradiated CB57BL/6J-recipient mice (***Figure 4—figure supplement 1***). Mice transplanted with bone marrow from CB_2-GFP mice (CB_2 -GFP BMT) or from C57BL/6J mice (C57BL/6J BMT) were exposed to a partial sciatic nerve ligation or a sham surgery and dorsal root ganglia were collected 14 days later. A significant infiltration of non-neuronal GFP+ cells was revealed in nerve injured CB_2-GFP BMT mice (~30 cells/mm^2, ***Figure 4A***, ***Figure 4—figure supplement 2***), indicating that CB_2 -expressing cells invaded the injured nerve. Immunostaining to identify these cell types revealed co-localization with macrophage and lymphocyte markers. Nearly 60% of infiltrating macrophages and around 40% of the lymphocytes were found to be GFP+ (***Figure 4B***, ***Figure 4C***, ***Figure 4—figure supplement 3***). Surprisingly, a significant percentage of neurons was also found to express GFP in CB_2-GFP BMT mice (***Figure 4D***). The percentage of GFP+ neurons was higher in nerve-injured mice (~4% of total neurons) than in sham-operated animals (~2%, ***Figure 4D***, ***Figure 4—figure supplement 4***). Since GFP could only come from bone-marrow transplanted cells, this finding suggests a transfer of CB_2 from bone-marrow derived cells to neurons. Hence, nerve injury facilitated the invasion of affected ganglia by CB_2-positive immune cells and promoted a neuronal GFP expression compatible with transfer of CB_2 from immune cells to neurons.

Lymphocyte involvement on JWH133 efficacy

The discovery of CB_2-expressing lymphocytes invading the dorsal root ganglia of nerve-injured mice prompted us to investigate the role of this cell type in spontaneous neuropathic pain. To answer this question, C57BL/6J mice were repeatedly treated with a control IgG or with an antibody targeting intercellular adhesion molecule 1 (ICAM1), a protein required for lymphocyte extravasation (***Labuz et al., 2009***). Mice under treatment with anti-ICAM1 or with the control IgG were exposed to JWH133 self-administration. Instead of reducing the intake of the CB_2 agonist, anti-ICAM1 significantly increased active nose poking to obtain i.v. JWH133 without altering the inactive nose poking (***Figure 5A***, ***Figure 5—figure supplement 1A***), suggesting increased spontaneous pain. This result is in agreement with previous works showing protection against chronic inflammatory and

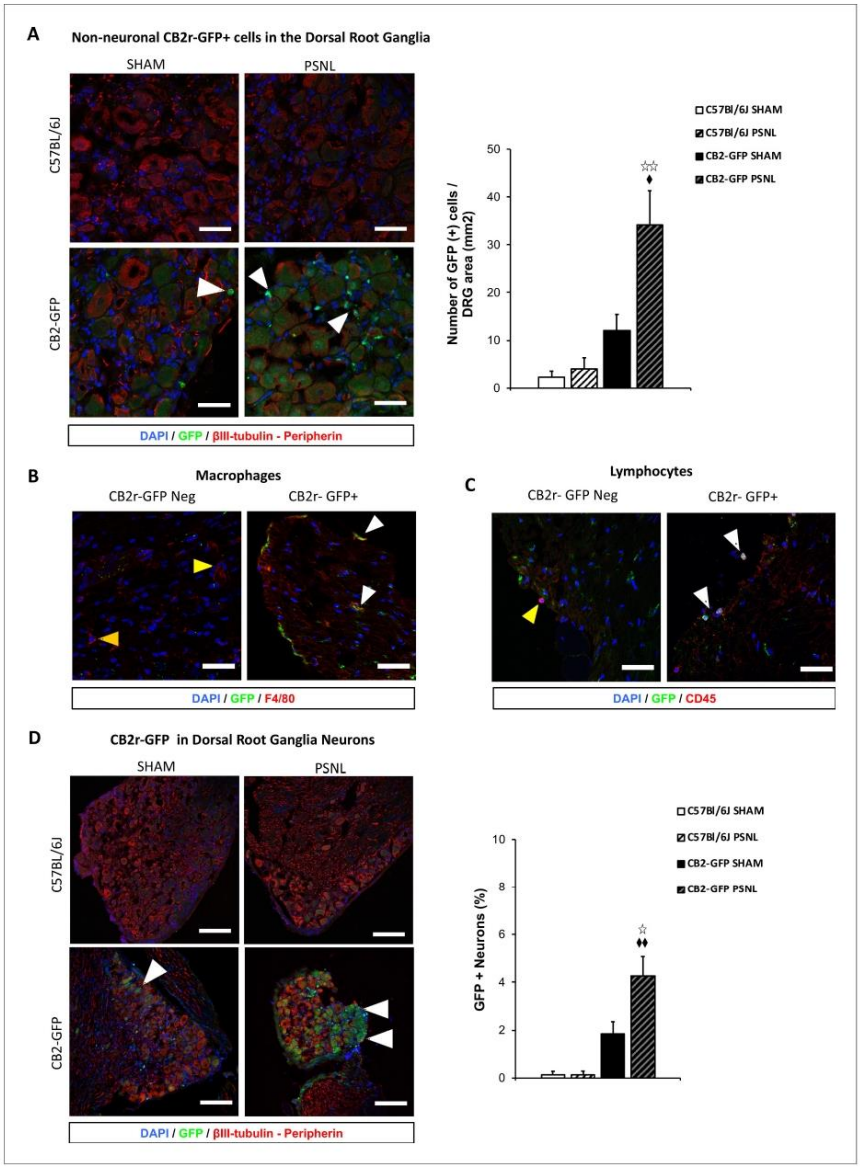

Figure 4. CB_2 receptor-GFP immune cells infiltrate the dorsal root ganglia of the injured nerve and GFP from bone-marrow-derived cells is also found inside peripheral neurons. The figure shows images of L3-L5 dorsal root ganglia from sham (SHAM) or nerve-injured mice (PSNL) transplanted with bone marrow cells from CB_2 GFP BAC mice (CB_2-GFP) or C57BL6/J mice (C57BL6/J). (**A, D**) Dorsal root ganglia sections stained with the nuclear marker DAPI (Blue), anti-GFP (Green), and neuronal markers anti-β-III tubulin and anti-peripherin (Red). (**A**) CB_2-GFP mice showed significant infiltration of GFP

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Figure 4 continued

+ bone-marrow-derived cells after the nerve injury, whereas sham or nerve-injured C57BL6/J mice did not show significant GFP immunorreactivity. Split channels in ***Figure 4—figure supplement 2***. (**B**) Co-localization of CB_2-GFP and the macrophage marker anti-F4/80. Co-staining with anti-GFP and anti-F4/80 revealed GFP+ (~60%) and GFP negative macrophages infiltrating the injured nerve. Split channels in ***Figure 4—figure supplement 3A***. (**C**) Co-staining with anti-GFP and anti-CD45 revealed GFP+ (~40%) and GFP-negative lymphocytes infiltrating the injured nerve. Split channels in ***Figure 4—figure supplement 3B***. (**D**) CB_2-GFP mice showed a percentage of GFP+ neurons that was enhanced with the nerve injury. Scale bar, 140 µm. Split channels in ***Figure 4—figure supplement 4***. Scale bar for B), C), D), 45 µm. Yellow arrows point to GFP negative cells and white arrows to GFP+ cells. A certain degree of image processing has been applied equally across the entire merged images for optimal visualization. N = 2–3 mice per group. Means and error bars representing SEM are shown. Stars represent comparisons vs. sham; diamonds vs. C57BL6/J. *p<0.05, **p<0.01, ***p<0.001. Flow cytometry of blood from CB_2-GFP and C57BL6/J mice in ***Figure 4—figure supplement 1***. Additional images of Sham and nerve-injured CB_2-GFP mice in ***Figure 4—figure supplements 5*** and ***6***. Specificity tests for Tyramide Signal Amplification in ***Figure 4—figure supplement 7***. Controls for antibody specificity in ***Figure 4—figure supplement 8***.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. CB_2 GFP cells in dorsal root ganglia of C57BL6/J nerve-injured mice after bone-marrow transplants from CB_2 GFP BAC mice.
Figure supplement 1. Bone marrow transplantation from CB_2 GFP BAC to C57BL6/J mice yields mice with peripheral blood cells expressing GFP.
Figure supplement 1—source data 1. Flow cytometry of blood from C57BL6/J mice transplanted with bone marrow from CB_2 GFP BAC mice.
Figure supplement 2. Non-neuronal CB_2 -GFP+ cells in the Dorsal Root Ganglia.
Figure supplement 3. Presence of CB_2 receptor-GFP in immune cells in the Dorsal Root Ganglia.
Figure supplement 4. CB_2-GFP in Dorsal Root Ganglia Neurons.
Figure supplement 5. Additional images of CB_2-GFP in Dorsal Root Ganglia Neurons.
Figure supplement 6. Higher magnification of merged images shown in ***Figure 4—figure supplement 5***.
Figure supplement 7. Tyramide signal amplification (TSA) for optimal visualization of GFP in the Dorsal Root Ganglia.
Figure supplement 8. Controls for antibody specificity in the Dorsal Root Ganglia.

neuropathic pain mediated by lymphoid cells (***Labuz et al., 2009***; ***Baddack-Werncke et al., 2017***). Interestingly, thermal and mechanical nociception before self-administration were similar in anti-ICAM1 and control IgG-treated mice (***Figure 5B***). After self-administration, the alleviation of thermal sensitivity was similar in control IgG and anti-ICAM1-treated mice (***Figure 5B***), but mice treated with anti-ICAM1 also showed an abolition of the antinociceptive effect of JWH133 on mechanical sensitivity (***Figure 5B***). This was evident in spite of the increased drug-taking behavior shown by mice treated with anti-ICAM1 (***Figure 5A***), which reveals decreased antinociceptive efficacy of JWH133 in these mice. On the contrary, anxiety-like behavior was similar in Control IgG and anti-ICAM1 mice (***Figure 5C***). To confirm an effect of the antibody treatment on lymphocyte infiltration, RT-PCR for white blood cell markers was performed in the dorsal root ganglia of mice subjected to the behavioral paradigm. As expected, a significant decrease in T cell markers CD2 and CD4 was observed in mice treated with anti ICAM-1 (***Figure 5D,T*** cell panel). Interestingly, anti ICAM-1 also showed a pronounced increase in B cell marker CD19 (***Figure 5D***) and no alteration of the macrophage marker C1q (***Figure 5D***). Hence, our results reveal that lymphoid cells are involved in spontaneous neuropathic pain and are also necessary for the antinociceptive effect of JWH133 on mechanical sensitivity.

Discussion

This work shows a protective function of CB_2 from neurons and lymphocytes on spontaneous neuropathic pain and the involvement of these cell populations in CB_2-induced antinociception, as revealed by increased self-administration of the CB_2 agonist JWH133 in mice defective in lymphocyte and neuronal CB_2. Previous works already demonstrated antinociceptive and emotional-like effects of CB_2 agonists in rodent models of acute and chronic pain (***Gutierrez et al., 2011***; ***Ibrahim et al., 2003***; ***Jafari et al., 2007***; ***La Porta et al., 2015***; ***Maldonado et al., 2016***). Our results provide evidence that the effect of the CB_2 agonist is sufficient to promote drug-taking behavior in nerve-injured mice for alleviation of spontaneous pain, but it is void of reinforcing effects in animals without pain, suggesting the absence of abuse liability. This absence of reinforcement adds value to the modulation of pain through CB_2 agonists, since current available agents for neuropathic pain treatment have reduced efficacy and often show addictive properties in humans and rodents (***Attal and Bouhassira, 2015***; ***Bonnet and Scherbaum, 2017***; ***Bura et al., 2018***; ***Finnerup et al., 2015***; ***Hipólito et al., 2015***; ***O'Connor et al., 2011***).

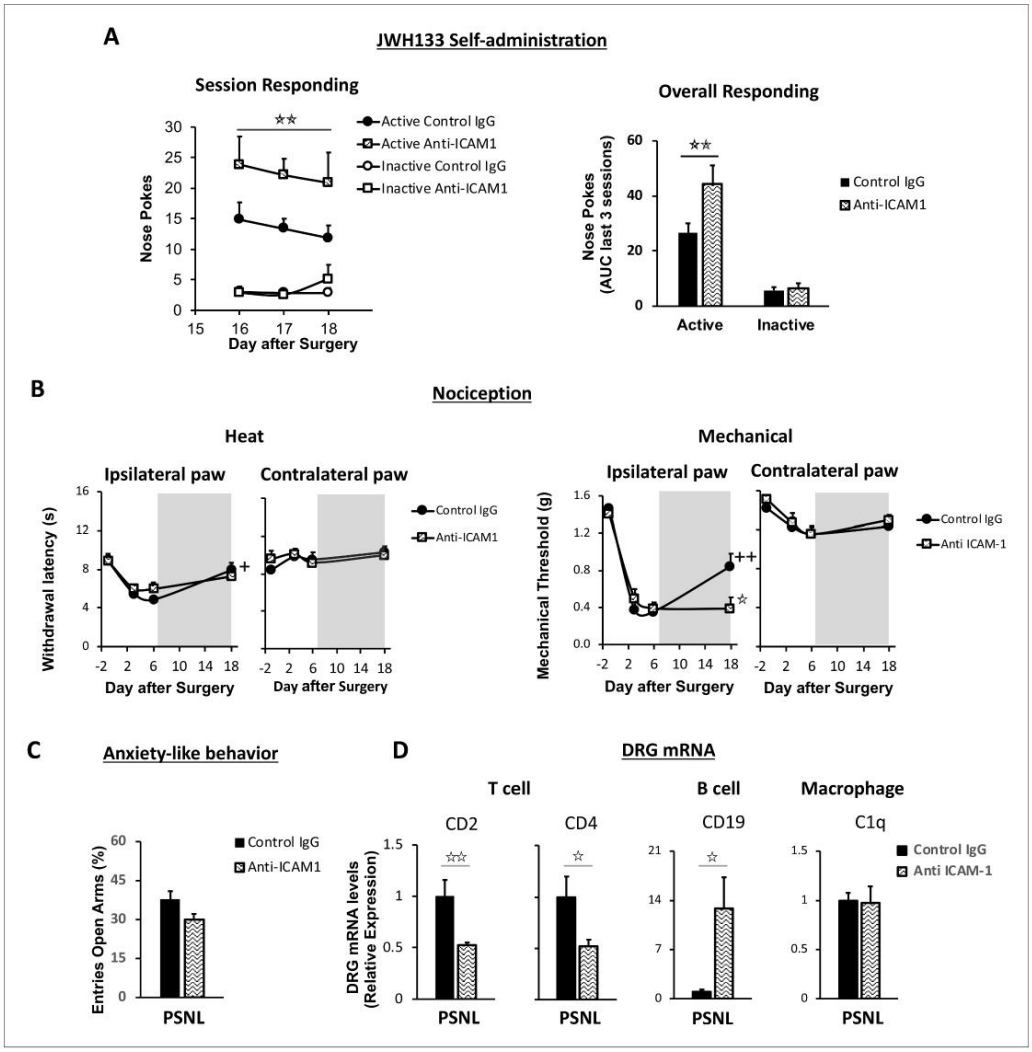

Figure 5. Lymphocytes modulate the effects of JWH133 on spontaneous pain and mechanical nociception. C57BL/6J mice were food-trained in Skinner boxes (Food training, 5 days), subjected to partial sciatic nerve ligation (PSNL, day 0), catheterized and exposed to high doses of the CB_2 agonist JWH133 (0.3 mg/kg/inf., days 7 to 18). Treatments with Anti-ICAM1 (an antibody that inhibits lymphocyte extravasation) or control IgG were given intraperitoneally once a day from day 0 until the end of self-administration. Nociceptive sensitivity to heat (Plantar) and mechanical (von Frey) stimulation was measured before and after nerve injury (−1,3,6,18), and anxiety-like behavior was evaluated at the end (day 19). Dorsal root ganglia were collected for mRNA analysis (**A**) Mice treated with anti-ICAM1 showed increased active responding for JWH133. (**B**) Thermal nociception after JWH133 self-administration was similar in mice treated with anti-ICAM1 or control IgG. Conversely, JWH133 effects on mechanical nociception were abolished by anti-ICAM1. (**C**) Anxiety-like behavior was similar in anti-ICAM1 and control IgG mice. (**D**) Levels of mRNA from T cell markers CD2 and CD4 were decreased in the dorsal root ganglia of anti-ICAM1 mice. Conversely, levels of B cell marker CD19 increased. Macrophage marker C1q was

Figure 5 continued on next page

Figure 5 continued

unaffected. N = 6–7 mice per group. Shaded areas represent drug self-administration. Mean and error bars representing SEM are shown. Stars represent comparisons vs. control IgG group; crosses indicate day effect. *p<0.05; **p<0.01; ***p<0.001.
The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. JWH133 self-administration, antinociception and anxiolytic-like effects in nerve-injured C57BL6/J mice treated with anti-ICAM1.
Figure supplement 1. JWH133 self-administration of nerve-injured mice treated with anti-ICAM1 or control IgG and food-maintained operant training before nerve injury and drug self-administration.
Figure supplement 1—source data 1. Operant training and full JWH133 self-administration in nerve-injured C57BL6/J mice treated with anti-ICAM1.

A previous work using the CB_2 agonist AM1241 showed drug-taking behavior and antinociception in nerve-injured rats (***Gutierrez et al., 2011***), although a recent multicenter study demonstrated off-target effects of this drug (***Soethoudt et al., 2017***). The disruption of JWH133 effects observed in constitutive knockout mice confirms that the relief of spontaneous pain and the effects reducing mechanical nociception and anxiety-like behavior are mediated by CB_2 stimulation. However, the CB_2 agonist partially preserved its effects promoting drug self-administration and relieving thermal hypersensitivity in CB2KO mice, suggesting that JWH133 may also act through other receptors. Our results with the nerve-injured TRPA1 knockout mice revealed that JWH133 preserves its efficacy inducing antinociception in the absence of this receptor. Therefore, TRPA1 does not seem to play a relevant role in the antinociceptive response induced by JWH133 in our model of neuropathic pain mice. Interestingly, TRPA1 deletion prevented the development of thermal hyperalgesia after the nerve injury in our study and formalin (0.5%) evoked nocifensive behaviors are also lost in the TRPA1 knockout (***McNamara et al., 2007***). Another possibility is a minor involvement of CB_1 receptor in JWH133 self-administration after the nerve injury, since this compound is a selective CB_2 agonist that exhibited 40-fold higher affinity for mouse CB_2 than for CB_1 receptor (***Soethoudt et al., 2017***). Our experiments were not designed to rule out this possible minor participation. However, the inactive responding in the operant self-administration sessions was similar after JWH133 or vehicle in nerve-injured mice, indicating an absence of primary CB_1-related side effects such as motor alteration that are classical effects observed in mice after the administration of CB_1 agonists (***Rodríguez de Fonseca et al., 1998***). Previous works using higher equivalent doses of a CB_2 agonist with similar potency (***Soethoudt et al., 2017***) showed also absence of motor impairment in nerve-injured rats (***Gutierrez et al., 2011***). Since JWH133 selectivity for human CB_2 vs. human CB_1 is higher (153-fold selectivity), we do not foresee any concern with CB1-related behavioral effects using similar molecules in humans.

Nerve-injured mice defective in neuronal CB_2 showed higher JWH133 intake than wild-type littermates, indicating persistence of drug effects and increased spontaneous pain when neurons do not express CB_2. Thus, increased self-administration suggests an enhanced affective-motivational component of pain and not reduced drug efficacy on this aspect, since nerve-injured C57BL6/J mice exposed to the low JWH133 dose did not show compensatory increased self–administration (***Figure 1***). Importantly, mechanical and thermal neuropathic hypersensitivity before drug self-administration were similar in neuronal knockouts and their wild-type littermates, which suggests different mechanisms of spontaneous pain and evoked nociception. In addition, mechanical nociception measured after JWH133 was more severe in neuronal CB_2 knockouts than in wild-type littermates, which indicates decreased JWH133 efficacy on mechanical antinociception. Several studies described the presence of CB_2 mRNA and functional CB_2 receptor in neuronal populations from different areas of the rain (***Stempel et al., 2016***; ***Zhang et al., 2014***). However, other works using targeted expression of fluorescent proteins under the control of the mouse gene *Cnr2* failed to describe CB_2 expression in neurons (***López et al., 2018***; ***Schmöle et al., 2015a***). Our results agree with a role of neuronal CB_2 during painful neuroinflammatory conditions, a setting that was not studied before in mice defective in neuronal CB_2. Although we cannot provide a precise localization of the neurons involved in the increased spontaneous and evoked pain of neuronal knockout mice, the similar JWH133 response of CB_2 Nav1.8 Cre+ mice lacking CB_2 in primary afferent neurons (Nav1.8-Cre+) and wild-type mice could indicate involvement of a different set of neurons or increased relevance of CB_2 from immune sources. Thermal hypersensitivity and anxiety-like behavior measured after self-administration was similar in neuronal knockouts and wild-type mice, which indicates involvement of non-

neuronal cell populations. However, it should also be considered that the neuronal knockout mice had higher JWH133 consumption. Thus, a possible lack of efficacy could also be present for thermal antinociception and inhibition of anxiety-like behavior. Although a neuronal involvement was found, CB_2 neuronal knockouts did not recapitulate the phenotype of mice constitutively lacking CB_2, suggesting additional cell types involved in the effects of CB_2agonists.

We investigated the effects of JWH133 promoting its own consumption and inducing antinociception and anxiolysis in CB_2 LysM-Cre+ mice, mainly lacking CB_2 in monocytes, the precursors of microglial cells. We did not observe a microglial participation in these pain-related phenotypes, which may be due to an incomplete deletion of CB_2 in microglia through LysM-driven Cre expression (**Blank and Prinz, 2016**). Previous studies in mice constitutively lacking CB_2 described an exacerbated spinal cord microgliosis after nerve injury (**Nozaki et al., 2018**; **Racz et al., 2008**), which suggested a relevant role of CB_2 controlling glial reactivity. Since spinal microgliosis participates in the increased pain sensitivity after a neuropathic insult and macrophages and microglia express CB_2, blunted effects of JWH133 were expected in microglial CB_2 knockouts. However, monocyte-derived cells did not seem to be involved in the analgesic effects mediated by the exogenous activation of CB_2 in these experimental conditions.

The immunohistochemical analysis of dorsal root ganglia from mice transplanted with bone marrow cells of CB_2GFP BAC mice (**Schmöle et al., 2015a**) revealed a pronounced infiltration of immune cells expressing CB_2 in the dorsal root ganglia after nerve injury. Macrophages and lymphocytes expressing CB_2 were found at a time point in which nerve-injured mice present mechanical and thermal hypersensitivity and self-administer compounds with demonstrated analgesic efficacy (**Bura et al., 2013**; **Bura et al., 2018**). Interestingly, GFP expression was also found in neurons, suggesting a transfer of CB_2 from peripheral immune cells to neurons. An explanation for this finding may come from processes of cellular fusion or transfer of cargo between peripheral blood cells and neurons (**Alvarez-Dolado et al., 2003**; **Ridder et al., 2014**). Bone-marrow-derived cells fuse with different cell types in a process of cellular repair that increases after tissue damage. These events may be particularly important for the survival of neurons with complex structures that would otherwise be impossible to replace (**Giordano-Santini et al., 2016**). Alternatively, extracellular vesicles drive intercellular transport between immune cells and neurons (**Budnik et al., 2016**). Earlier studies showed incidence of fusion events between bone-marrow-derived cells and peripheral neurons in a model of diabetic neuropathy (**Terashima et al., 2005**), and similar processes were observed in central neurons after peripheral inflammation (**Giordano-Santini et al., 2016**; **Ridder et al., 2014**). Functional contribution of these mechanisms to neuronal CB_2 expression has not yet been explored, although cargo transfer between immune cells and neurons could modify neuronal functionality and it could offer novel therapeutic approaches to modulate neuronal responses (**Budnik et al., 2016**). Hence, CB_2 coming from white blood cells and present in neurons could be significant modulators of spontaneous neuropathic pain and may be contributors to the analgesic effect of CB_2 agonists.

Our results suggest participation of lymphoid cells on spontaneous neuropathic pain, but not on basal neuropathic hypersensitivity, highlighting possible differences on the pathophysiology of these nociceptive manifestations. In addition, lymphoid cells were essential for the effects of JWH133 alleviating mechanical sensitivity after a nerve injury. These hypotheses were evaluated by using anti-ICAM1 antibodies that impair lymphocyte extravasation. Previous studies revealed that anti-ICAM1 treatment inhibited opioid-induced antinociception in a model of neuropathic pain (**Celik et al., 2016**; **Labuz et al., 2009**). According to the authors, stimulation of opioid receptors from the immune cells infiltrating the injured nerve evoked the release of opioid peptides that attenuated mechanical hypersensitivity. Lymphocyte CB_2 could also be involved in the release of leukocyte-derived pain-modulating molecules. Experiments assessing the function of ICAM1 (**Celik et al., 2016**; **Deane et al., 2012**; **Labuz et al., 2009**) showed the participation of this protein on lymphocyte extravasation. In agreement, we observed a decrease of T cell markers in the dorsal root ganglia of mice receiving anti-ICAM1. Anti-ICAM1 treatment also increased the mRNA levels of the B cell marker CD19 in the dorsal root ganglia. Since ICAM1-interacting T cells show activity limiting B cell populations (**Deane et al., 2012**; **Zhao et al., 2006**), it is likely that the absence of T cells in the nervous tissue increased infiltration of B lymphocytes. B cells are involved in the severity of neuroinflammatory processes and have been linked to pain hypersensitivity (**Huang et al., 2016**; **Jiang et al., 2016**; **Li et al., 2014**; **Zhang et al., 2016**). Interestingly, CB_2 receptors restrict glucose and energy supply of B cells (**Chan et al., 2017**), which may alter their cytokine production as

previously described for macrophages and T cells. However, the participation of CB_2 from B cells on neuropathic pain has not yet been established. Our results indicate an increase in JWH133 consumption that could be driven by an increased infiltration of B cells. Overall, the results with the ICAM-1 experiment suggest a relevant participation of lymphoid CB_2 on painful neuroinflammatory responses. Our data also underscore the interest of investigating the role of lymphoid cells in brain regions involved in pain, anxiety or negative reinforcement during chronic neuroinflammatory processes.

While we identify CB_2-expressing neurons and lymphocytes as cellular entities involved in spontaneous and evoked neuropathic pain, the efficacy of the CB_2 agonist eliciting its own self-administration to alleviate pain was only disrupted in constitutive CB_2 knockout mice. These results indicate that the cell types involved in the negative reinforcement induced by JWH133 were suboptimally targeted in our experimental conditions, probably because different cell types expressing CB_2 are involved in this phenotype. Vascular cells may represent alternative participants of this behavior since JWH133 showed local vasodilatory effects (***McDougall et al., 2008***) and endothelial functional CB_2 receptor was found in cerebral microvasculature (***Ramirez et al., 2012***; ***Onaivi et al., 2012***).

In summary, the contribution of neurons and lymphocytes to the effects of CB_2 agonists on spontaneous and evoked pain suggests a coordinated response of both cell types after the nerve injury. CB_2-expressing lymphocytes could participate in pain sensitization through release of pain-related molecules and the observed responses are also compatible with transfer of CB_2 between immune cells and neurons. Hence, bone-marrow-derived cells may provide a source of functional CB_2 that was not considered before and could clarify the controversial presence of these receptors in neurons. Nociceptive and affective manifestations of chronic neuropathic pain are therefore orchestrated through neuronal and immune sites expressing CB_2, highlighting the functional relevance of this cannabinoid receptor in different cell populations.

Our results on operant JWH133 self-administration depict CB_2 agonists as candidate analgesics for neuropathic conditions, void of reinforcing effects in the absence of pain. These pain-relieving effects involve the participation of CB_2 from neurons and lymphocytes preventing the neuroinflammatory processes leading to neuropathic pain. Therefore, CB_2 agonists would be of interest for preventing neuropathic pain development and the potential trials to evaluate this effect should consider starting CB_2 agonist treatment before or shortly after the induction of neuropathic insults, as in our study, in contrast to the treatment strategies used in previous clinical trials. The identification of a cannabinoid agonist simultaneously targeting the behavioral traits and the multiple cell types involved in the pathophysiology of chronic neuropathic pain acquires special relevance in a moment in which the absence of efficient analgesics void of abuse liability has become a major burden for public health.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (*Mus musculus*, male)	C57BL/6J	Charles Rivers, France	RRID:IMSR_JAX:000664	
Genetic reagent (*M. musculus*)	CB_2 KO	Institute of Molecular Psychiatry, University of Bonn, Germany	RRID:MGI:2663848	***Buckley et al., 2000*** PMID:10822068 (male)
Genetic reagent (*M. musculus*)	SynCre+-Cnr2$^{fl/fl}$:: Cnr2$^{fl/fl}$	Institute of Molecular Psychiatry, University of Bonn, Germany		(C57BL/6J background, male)
Genetic reagent (*M. musculus*)	LysMCre+-Cnr2$^{fl/fl}$:: Cnr2$^{fl/fl}$	Institute of Molecular Psychiatry, University of Bonn, Germany		(C57BL/6J background, male)
Genetic reagent (*M. musculus*)	Nav1.8Cre+-Cnr2$^{fl/fl}$:: Cnr2$^{fl/fl}$	Institute of Molecular Psychiatry, University of Bonn, Germany		(C57BL/6J background, male)

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*M. musculus*)	TRPA1 KO	Universidad Miguel Hernández, Spain	RRID:MGI:3625358	***Kwan et al., 2006***. PMID:16630838 (male)
Strain, strain background (*M. musculus*, male)	C57BL/6JRccHsd	Universidad Miguel Hernández, Spain		Envigo
Antibody	Anti-mouse ICAM-1 (Hamster, monoclonal, clone 3e2)	BD Biosciences, USA	550287	(150 µg/day i.p.)
Antibody	IgG from rabbit serum (Unconjugated)	Sigma-Aldrich, Germany	I5006	(150 µg/day i.p.)
Antibody	Allophycocyanin-conjugated anti-mouse CD11b (Monoclonal)	eBioscience, USA	cn.17–0112	Flow cytometry (1:300)
Antibody	Phycoerythrin -conjugated anti-mouse B220 (Monoclonal)	eBioscience, USA	cn.12–0452	Flow cytometry (1:100)
Antibody	Phycoerythrin/cyanine-conjugated anti-mouse CD3 (Monoclonal)	BioLegend, USA	cn.100320	Flow cytometry (1:100)
Antibody	Rabbit anti-peripherin (Polyclonal)	Thermo Fisher, USA	PA3-16723	IHC (1:200)
Antibody	Rabbit anti-GFP antibody (Polyclonal)	Thermo Fisher, USA	A11122	IHC (1:2000)
Antibody	Rabbit anti-β-III tubulin (Polyclonal)	Abcam, UK	Ab18207	IHC (1:1000)
Antibody	Rat anti-CD45R/B220 APC antibody (Monoclonal, Clone RA3- 6B2)	Biolegend, USA	103229	IHC (1:500)
Antibody	Rat anti-F4/80 antibody (Monoclonal, Clone A3-1)	Biorad, USA	MCA497GA	IHC (1:500)
Antibody	Anti-rabbit poly-HRP-conjugated (Polyclonal)	Thermo Fisher, USA	Tyramide Superboost Kit, B40922	IHC (1X)
Antibody	Goat anti-rabbit Alexa Fluor A555 (Polyclonal)	Abcam, UK	Ab150078	IHC (1:1000)
Antibody	Goat anti-rat Alexa Fluor A555 (Polyclonal)	Abcam, UK	Ab150158	IHC (1:1000)
Chemical compound, drug	JWH133	Tocris, UK	TO-1343	CB_2 receptor agonist
Chemical compound, drug	Sodium thiopental	Braun medical, Spain	635573	
Chemical compound, drug	Isoflurane	Virbac, Spain	575837–4	
Chemical compound, drug	Paraformaldehyde	Merck Millipore, Germany	104005	
Chemical compound, drug	DAPI Fluoromount-G mounting media	SouthernBiotech, USA	0100–20	
Commercial assay or kit	RNeasy Micro kit	Qiagen, Germany	74004	
Commercial assay or kit	Omniscript reverse transcriptase	Qiagen, Germany	205111	
Commercial assay or kit	Tyramide Superboost Kit	Thermo Fisher, USA	B40922	

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Software, algorithm	FACSDiva version 6.2	BD biosciences, USA	RRID:SCR_001456	
Software, algorithm	Fiji	Wayne Rasband, USA	RRID:SCR_002285	
Software, algorithm	IBM SPSS 19	IBM Corporation, USA	RRID:SCR_002865	
Software, algorithm	STATISTICA 6.0	StatSoft, USA	RRID:SCR_014213	

Animals

C57BL/6J male mice were purchased from Charles River Laboratories (L'Arbresle, France), and CB_2 knockout male mice defective in the *Cnr2* gene were bred in the Institute of Molecular Psychiatry (University of Bonn, Bonn, Germany). CB_2 constitutive knockouts were bred from heterozygous parents and their wild-type littermates were used as controls. Neuron and microglia/macrophage-specific conditional CB_2 knockout mice were generated as previously described (***Stempel et al., 2016***). Briefly, mice expressing Cre recombinase under the *Synapsin I* promoter (Syn-Cre+), mice expressing Cre recombinase inserted into the first coding ATG of the *Lyz2* gene (LysM-Cre+) and mice expressing Cre under the promoter of the gene *Scn10a* that codes for Nav1.8 voltage-gated sodium channels (Nav1.8-Cre+) were crossed with *Cnr2* floxed animals ($Cnr2^{fl/fl}$ mice) to obtain Cre+::$Cnr2^{fl/fl}$ mice. These F1 mice (Syn-Cre+-$Cnr2^{fl/-}$, LysM-Cre+-$Cnr2^{fl/-}$ and Nav1.8-Cre+-$Cnr2^{fl/-}$) were backcrossed to $Cnr2^{fl/fl}$ mice to generate mice $Cnr2^{fl/fl}$ and heterozygous for Cre (Cre+-$Cnr2^{fl/fl}$). Syn-Cre+-$Cnr2^{fl/fl}$ (Syn-Cre+), LysM-Cre+-$Cnr2^{fl/fl}$ (LysM-Cre+) and Nav1.8-Cre+-$Cnr2^{fl/fl}$ (Nav1.8-Cre+) mice were selected and further backcrossed to $Cnr2^{fl/fl}$ mice to produce experimental cohorts Syn-Cre+-$Cnr2^{fl/fl}$::$Cnr2^{fl/fl}$, LysM-Cre+-$Cnr2^{fl/fl}$::$Cnr2^{fl/fl}$ and Nav1.8-Cre+-$Cnr2^{fl/fl}$::$Cnr2^{fl/fl}$ containing 50% conditional knockout animals (also referred to as neuronal knockouts or Syn-Cre+, microglial knockouds or LysM-Cre+ and Nav1.8 knockouts or Nav1.8-Cre+) and 50% littermate control animals (referred to as Cre Negative mice throughout the study). Mice defective in the *Trpa1* gene (TRPA1 knockouts, ***Kwan et al., 2006***) and their respective control mice (C57BL/6JRccHsd) were bred in the animal facility at Universidad Miguel Hernández (UMH, Elche, Alicante, Spain). For bone-marrow transplantation studies, 2 CB_2-GFP BAC mice (***Schmöle et al., 2015a***) or C57BL/6J mice were used as donors and C57BL/6J mice were used as recipient mice. All mice had a C57BL/6J genetic background. The behavioral experimental sequence involving operant self-administration and assessment of nociceptive and anxiety-like behavior was repeated three times in the experiments assessing the effects of JWH133 doses (***Figure 1***) and 4 and 5 times in the experiments evaluating constitutive and conditional knockout mice, respectively (***Figures 2*** and ***3***). The experiments involving bone-marrow transplantation and lymphocyte depletion were performed once. Sample size was based on previous studies in our laboratory using comparable behavioral approaches (***Bura et al., 2013***; ***Bura et al., 2018***; ***La Porta et al., 2015***).

The behavioral experiments were conducted in the animal facilities at Universitat Pompeu Fabra (UPF)-Barcelona Biomedical Research Park (PRBB; Barcelona, Spain) and UMH (Elche, Alicante, Spain). Mice were housed in temperature (21 ± 1˚C) and humidity-controlled (55 ± 10%) rooms. For the self-administration experiments, animals were handled during the dark phase of a 12 hr light/dark reverse cycle (light off at 8:00 a.m., light on at 8:00 p.m.). Before starting the experimental procedure, mice were single housed and handled/habituated for 7 days. Food and water were available ad libitum except during the training period for food-maintained operant behavior, when mice were exposed to restricted diet for 8 days. Animal handling and experiments were in accordance with protocols approved by the respective Animal Care and Use Committees of the PRBB, Departament de Territori i Habitatge of Generalitat de Catalunya, UMH and the Institute of Molecular Psychiatry and were performed in accordance with the European Communities Council Directive (2010/63/EU). Whenever possible, animals were randomly assigned to their experimental condition, and experiments were performed under blinded conditions for surgery and pharmacological treatment (***Figure 1***), genotype (***Figures 2*** and ***3***), bone-marrow transplant and surgery (***Figure 4***), and antibody treatments (***Figure 5***).

Drugs

JWH133 (Tocris, Bristol, UK) was dissolved in vehicle solution containing 5% dimethyl sulfoxide (Scharlab, Sentmenat, Spain) and 5% cremophor EL (Sigma-Aldrich, Steinheim, Germany) in sterilized water and filtered with a 0.22 µm filter (Millex GP, Millipore, Cork, Ireland). JWH133 was self-administered intravenously (i.v.) at 0.15 or 0.3 mg/kg/infusion in volume of 23.5 µl per injection. In the additional experiments assessing nociceptive behavior in Nav1.8-Cre+ and TRPA1 knockout mice, JWH133 was diluted in a vehicle composed of 5% ethanol (Alcoholes Montplet, Barcelona, Spain), 5% Cremophor EL, and 90% saline (0.9% NaCl; Laboratorios Ern, Barcelona, Spain) to be administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Thiopental (Braun Medical, Barcelona, Spain) was dissolved in saline and administered through the implanted i.v. catheter at 10 mg/kg in a volume of 50 µl.

Antibody treatment

Anti-ICAM-1 antibody (clone 3E2; 150 µg; BD Biosciences, Franklin Lakes, NJ) and control rabbit IgG (150 µg; Sigma-Aldrich) were dissolved in saline up to a volume of 300 µl as previously reported (***Labuz et al., 2009***), and administered i.p. once a day from the day of the surgery to the last self-administration day.

Operant self-administration

Mice were first trained for operant food self-administration to facilitate subsequent drug self-administration, as previously described (***Bura et al., 2018***). Briefly, mice were food-restricted for 3 days to reach 90% of their initial weight. Then, mice were trained in skinner boxes (model ENV-307A-CT, Med Associates Inc, Georgia, VT) for 5 days (1 hr session per day) to acquire an operant behavior to obtain food pellets (***Figure 1—figure supplement 1B***, ***Figure 2—figure supplement 1B***, ***Figure 3—figure supplement 1B***, ***Figure 5—figure supplement 1B***). A fixed ratio 1 schedule of reinforcement (FR1) was used, that is 1 nose-poke on the active hole resulted in the delivery of 1 reinforcer together with a light-stimulus for 2 s (associated cue). Nose poking on the inactive hole had no consequence. Each session started with a priming delivery of 1 reinforcer and a timeout period of 10 s right after, where no cues and no reward were provided following active nose-pokes. Food sessions lasted 1 hr or until mice nose-poked 100 times on the active hole, whichever happened first. After the food training, mice underwent a partial sciatic nerve ligation (PSNL) or a sham surgery, and 4 days later an i.v. catheter was implanted in the right jugular vein to allow drug delivery. Mice started the drug self-administration sessions 7 days after the PSNL/sham surgery. In these sessions, the food reinforcer was substituted by drug/vehicle infusions. Self-administration sessions were conducted during 12 consecutive days, and mice received JWH133 (0.15 or 0.3 mg/kg) or vehicle under FR1 (***Figure 1—figure supplement 1B***, ***Figure 2—figure supplement 1B***, ***Figure 3—figure supplement 1B***, ***Figure 5—figure supplement 1B***). Sessions lasted 1 hr or until 60 active nose-pokes. Active and inactive nose-pokes were recorded after each session and discrimination indices were calculated as the difference between the nose pokes on the active and the inactive holes, divided by the total nose pokes. Data from the last three drug self-administration sessions was used for statistical analysis to exclude interference with food-driven operant behavior.

Partial Sciatic Nerve Ligation

Mice underwent a partial ligation of the sciatic nerve at mid-thigh level to induce neuropathic pain, as previously described (***Malmberg and Basbaum, 1998***) with minor modifications. Briefly, mice were anaesthetized with isoflurane (induction, 5% V/V; surgery, 2% V/V) in oxygen and the sciatic nerve was exposed at the level of the mid-thigh of the right hind leg. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the cranial side of the sciatic nerve using a 9–0 non-absorbable virgin silk suture (Alcon Cusí SA, Barcelona, Spain) and leaving the rest of the nerve untouched. The muscle was then stitched with 6–0 silk (Alcon Cusí), and the incision was closed with wound clips. Sham-operated mice underwent the same surgical procedure except that the sciatic nerve was not ligated.

Catheterization

Mice were implanted with indwelling i.v. silastic catheter, as previously reported (**Soria et al., 2005**). Briefly, a 5.5 cm length of silastic tubing (0.3 mm inner diameter, 0.64 mm outer diameter; Silastic, Dow Corning Europe, Seneffe, Belgium) was fitted to a 22-gauge steel cannula (Semat Technical Ltd., Herts, UK) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Wehrheim, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain).

Nociception

Sensitivity to heat and mechanical stimuli were used as nociceptive measures of neuropathic pain. Ipsilateral and contralateral hind paw withdrawal thresholds were evaluated the day before, 3 and 6 days after the nerve injury, as well as after the last self-medication session on day 18. Heat sensitivity was assessed by recording the hind paw withdrawal latency in response to radiant heat applied with the plantar test apparatus (Ugo Basile, Varese, Italy) as previously reported (**Hargreaves et al., 1988**). Punctate mechanical sensitivity was quantified by measuring the withdrawal response to von Frey filament stimulation through the up–down paradigm, as previously reported (**Chaplan et al., 1994**). Filaments equivalent to 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g were used, applying first the 0.4 g filament and increasing or decreasing the strength according to the response. The filaments were bent and held for 4–5 s against the plantar surface of the hind paws. Clear paw withdrawal, shaking or licking was considered a nociceptive-like response. Four additional filaments were applied since the first change of response (from negative to positive or from positive to negative), once each time. The sequence of the last six responses was used to calculate the withdrawal threshold following the method described by **Dixon, 1965**.

Anxiety-like behavior

Anxiety-like behavior was evaluated with an elevated plus maze made of Plexiglas and consisting of four arms (29 cm long x 5 cm wide), two open and two closed, set in cross from a neutral central square (5 × 5 cm) elevated 40 cm above the floor. Light intensity in the open and closed arms was 45 and 5 lux, respectively. Mice were placed in the neutral central square facing 1 of the open arms and tested for 5 min. The percentage of entries and time spent in the open and closed arms was determined.

RNA extraction and reverse transcription

Ipsilateral L3-L4 dorsal root ganglia from mice of the ICAM-1 experiment were collected on day 20 after the PSNL. Samples were rapidly frozen in dry ice and stored at −80˚C. Isolation of total RNA was performed using the RNeasy Micro kit (Qiagen, Stokach, Germany) according to the manufacturer's instructions. Total RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Montchanin, DE). RNA quality was determined by chip-based capillary electrophoresis using an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA). Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen) at 37˚C for 60 min.

Quantitative real-time PCR analysis

The qRT-PCR reactions were performed using Assay-On-Demand TaqMan probes: Hprt1 Mm01545399_m1, CD2 Mm00488928 m1, CD4 Mm00442754_m1, CD19 Mm00515420_m1, C1q Mm00432162_m1 (Applied Biosystems, Carlsbad, CA) and were run on the CFX96 Touch Real-Time PCR machine (BioRad, Hercules, CA). Each template was generated from individual animals, and amplification efficiency for each assay was determined by running a standard dilution curve. The expression of the Hprt1 transcript was quantified at a stable level between the experimental groups to control for variations in cDNA amounts. The cycle threshold values were calculated automatically by the CFX MANAGER v.2.1 software with default parameters. RNA abundance was calculated as 2−(Ct). Levels of the target genes were normalized against the housekeeping gene, Hprt1, and compared using the ΔΔCt method (**Livak and Schmittgen, 2001**).

Bone marrow transplantation

C57BL/6J mice received bone marrow from CB_2-GFP BAC or C57BL/6J male mice. G-irradiation of C57BL/6J recipient male mice (9.5 Gy) was performed in a 137Cs-g IBL 437 C H irradiator (Schering CIS Bio international) at 2.56 Gy/min rate in order to suppress their immune response. Afterwards, approximately 5×10^5 bone marrow cells collected from donors (CB_2-GFP BAC or C57BL/6J) and transplanted through the retro-orbital venous sinus of the recipient mice. Irradiated mice were inspected daily and were given 150 ml of water with enrofloxacin at 570 mg/l and pH 7.4 (Bayer, Germany) for 30 days to reduce the probability of infection from opportunistic pathogens. Peripheral blood samples (150 µl) were collected by tail bleeding into a tube with 0.5 M EDTA solution to evaluate immune system recovery through flow cytometry 4, 8 and 12 weeks after the bone marrow transplantation.

Flow cytometry

For the analyses of hematopoietic cells, a hypotonic lysis was performed to remove erythrocytes. 50 µl of blood was lysed using 500 µl of ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Lonza, Walkersville) 10 min at room temperature. After the erythrocytes lysis, two washes with PBS were performed prior the incubation with the antibodies for 30 min at 4˚C. Cells were stained with the following fluorochrome-coupled antibodies: Allophycocyanin (APC)-conjugated anti-mouse CD11b (1:300; cn.17–0112 eBioscience, USA) to label myeloid cells, phycoerythrin (PE)-conjugated anti-mouse B220 (1:100; cn.12–0452, eBioscience, USA) for B lymphocytes and phycoerythrin/cyanine (PE/Cy7)-conjugated anti-mouse CD3, 1:100; cn.100320, BioLegend, USA) for T lymphocytes. Immunofluorescence of labeled cells was measured using a BD LSR II flow cytometer. Dead cells and debris were excluded by measurements of forward- versus side-scattered light and DAPI (4′,6-diamino-2-phenylindole) (Sigma) staining. Gates for the respective antibodies used were established with isotype controls and positive cell subset controls. Data analysis was carried out using FACSDiva version 6.2 software (BD biosciences).

Immunohistochemistry

Mice were sacrificed 2 weeks after the PSNL/sham surgery and L3-L5 dorsal root ganglia were collected to quantify GFP+ cells in mice transplanted with bone marrow cells of CB_2-GFP or C57BL6/J mice. Ganglia were freshly extracted and fixed in 4% paraformaldehyde during 25 min at 4˚C. After 3×5 min washes with phosphate buffered saline (PBS) 0.1 M (pH 7.4), were preserved overnight in a 30% sucrose solution in PBS 0.1 M containing sodium azide 0.02%. 24 hr later, ganglia were embedded in molds filled with optimal cutting temperature compound (Sakura Finetek Europe B.V., Netherlands) and frozen at −80˚C. Samples were sectioned with a cryostat at 10 µm, thaw-mounted on gelatinized slides and stored at −20˚C until use. Dorsal root ganglia sections were treated 1 hr with 0.3 M glycine, 1 hr with oxygenated water 3% (Tyramide Superboost Kit, B40922, Thermo Fisher, USA) and, after 3×5 min washes with PBS 0.01 M, 1 hr with blocking buffer. Samples were incubated 16 hr at room temperature with rabbit anti-GFP (1:2000, A11122, Thermo Fisher, USA) antibody. After 3×10 min washes with PBS 0.01 M, sections were incubated with anti-rabbit poly-HRP-conjugated secondary antibody for 1 hr and washed 4×10 min. Alexa Fluor tyramide reagent was applied for 10 min and then the Stop Reagent solution for 5 min (Tyramide Superboost Kit). Afterwards samples were incubated 2 hr at room temperature with primary antibodies diluted in blocking buffer (PBS 0.01 M, Triton X-100 0.3%, Normal Goat Serum 10%). The following primary antibodies were used: rabbit anti-peripherin (1:200, PA3-16723, Thermo Fisher, USA), rabbit anti-β-III tubulin (1:1000, ab18207, Abcam, UK), rat anti-CD45R/B220 APC (1:500, Clone RA3-6B2, 103229, Biolegend, USA) and rat anti-F4/80 (1:500, Clone A3-1, MCA497GA, Biorad, USA). After 3×5 min washes, all sections were treated with goat secondary antibodies from Abcam (UK) for 1 hr at room temperature: anti-rabbit Alexa Fluor 555 (1:1000, ab150078) and anti-rat Alexa Fluor 555 (1:1000, ab150158). Samples were then washed with PBS 0.01 M and mounted with 24×24 mm coverslips (Brand, Germany) using Fluoromount-G with DAPI (SouthernBiotech, USA).

Microscope image acquisition and processing

Confocal images were taken with a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) on a DM6000 stand using 20×0.7 NA Air and 63×1.4 NA Oil Immersion Plan

Apochromatic lenses. Leica Application Suite Advanced Fluorescence software (Leica Microsystems, Mannheim, Germany) was used to acquire the images and DAPI, Alexa 488 and Alexa 555 channels were taken sequentially. Images of DAPI were taken with 405 nm excitation and emission detection between 415 and 480 nm; images of Alexa 488 were taken with 488 nm excitation and emission detection between 495 and 540 nm; and images of Alexa 555 were taken with 543 nm excitation and emission detection between 555 and 710 nm. Room temperature was kept at 22 ± 1˚C during all imaging sessions. All images were equally processed and quantified with Fiji software (National Institutes of Health, USA). To determine the percentage of dorsal root ganglia area occupied by GFP (+) neurons auto-threshold ('Otsu') was set between 0–50 in all images and then converted to mask. Afterwards, operations included Close, Fill holes and Watershed neurons for separation and particles between 100–100000 pixel units and circularity 0.2–1.0 were counted. To analyze the number of GFP+ cells per dorsal root ganglia area, background was subtracted from all images (rolling = 5), set to an auto-threshold ('Default') between 0–70 and converted to mask. Particles considered GFP+ cells were nucleated, 7–100 microns^2 and 0.9–1.0 circularity.

Statistical analysis

Self-administration and nociceptive behavioral data were analyzed using a linear mixed model with three (surgery, day and dose) or two factors (day and genotype or antibody treatment) and their interactions. For the covariance structure of the repeated measures, a diagonal matrix was chosen. Bonferroni post hoc analysis was performed when pertinent. Areas Under the Curve (AUCs) of time-courses for operant responding were analyzed using two-way analysis of variance (ANOVA). Active and inactive responses were analyzed taking into account surgery and dose effects in the dose-response experiments, and active/inactive and genotype or antibody treatment in the knockout and antibody experiments. Anxiety-like behavior was analyzed using two-way ANOVA (surgery and dose for dose-response experiments), one-way ANOVA (genotype of conditional knockouts) or t-tests (constitutive knockout and antibody treatment), followed by Bonferroni adjustments when required. Mechanical and thermal thresholds in Nav1.8 and TRPA1 knockout mice treated with JWH133 were analyzed using three-way repeated measures ANOVA with surgery and genotype as between-subject factors and treatment as within-subject factor, followed by Bonferroni post-hoc test when appropriate. Immunohistochemistry of bone marrow-transplanted mice was analyzed using the Bonferroni-Dunn's test to adjust for multiple comparisons after multiple t-tests, and qPCR results after antibody treatments were compared with t-tests. IBM SPSS 19 (SPSS Inc, Chicago, IL) and STATISTICA 6.0 (StatSoft, USA) software were used to analyze the data, and differences were considered statistically significant when p value was below 0.05. All experimental data and statistical analyses of this study are included in the manuscript and its supplementary files. Raw data and results of statistical analyses are provided in the respective Source Data Files and their containing data sheets.

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David Cabañero, Conceptualization, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing - original draft, Writing - review and editing; Angela Ramírez-López, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing - review and editing; Eva Drews, Anne Schmöle, David M Otte, Resources, Validation, Methodology; Agnieszka Wawrzczak-Bargiela, Validation, Investigation, Visualization, Methodology; Hector Huerga Encabo, Validation, Investigation, Methodology; Sami Kummer, Validation, Methodology; Antonio Ferrer-Montiel, Resources, Writing - review and editing; Ryszard Przewlocki, Resources, Formal analysis, Supervision, Funding acquisition, Investigation, Visualization, Methodology, Writing - review and editing; Andreas Zimmer, Resources, Supervision, Validation, Investigation, Methodology, Writing - review and editing; Rafael Maldonado, Conceptualization, Resources, Data curation, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Project administration, Writing - review and editing

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Ethics

Animal experimentation: Animal handling and experiments were in accordance with protocols approved by the respective Animal Care and Use Committees of the PRBB, Departament de Territori i Habitatge of Generalitat de Catalunya and the Institute of Molecular Psychiatry and were performed in accordance with the European Communities Council Directive (2010/63/EU).

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Supplementary files

- Transparent reporting form

Data availability

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