OREXIN RECEPTORS AS THERAPEUTIC TARGETS FOR ADDICTION AND ANXIETY DISORDERS

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Abstract

Orexin-A and orexin-B (also known as hypocretin-1 and hypocretin-2) are two neuropeptides exclusively expressed by a small subpopulation of neurons located within the lateral hypothalamic area. These neuromodulators were originally thought to specifically mediate feeding and promote wakefulness. Nevertheless, it is now clear that they also participate in other behavioural and physiological processes, particularly those under high motivational or emotional circumstances, including reward occasions or exposure to threats. Growing evidence indicates that orexins might be involved in dysfunctional processing of these situations, highlighting their possible contribution to certain neuropsychiatric conditions such as drug addiction and anxiety disorders. By the use of behavioural and biochemical studies, the present thesis examines the role of orexin transmission in the pharmacological and reinforcing effects of cannabinoids, which represent important contributing factors for cannabis addiction. We report that orexins modulate hypothermic, antinociceptive and anxiolytic-like effects of Δ^9-tetrahydrocannabinol through orexin receptor-2, whereas they contribute to the reinforcing effects of the synthetic cannabinoid WIN55,212-2 through orexin receptor-1 signalling, in part through dopamine-dependent mechanisms. On the other hand, we have investigated the influence of orexins in fear memory processing, a pivotal component of diverse anxiety disorders. We confirmed the role of both orexin receptors in the consolidation of fear memories, and we established that orexin transmission, through orexin receptor-1 stimulation, hinders the extinction of aversive memories, probably due to its influence on the communication between the amygdala and the prefrontal cortex. The findings of the present thesis reveal the therapeutic potential of orexin receptor-1 blockade as a novel target to prevent cannabis addiction, as well as to alleviate abnormal fear retention in anxiety disorders such as posttraumatic stress disorder and phobias.

Resumen

La orexina-A y la orexina-B (también conocidas como hipocretina-1 e hipocretina-2) son dos neuropéptidos expresados exclusivamente por una pequeña población de neuronas localizadas en el área hipotalámica lateral. Estos neuromoduladores se relacionaron inicialmente con la regulación de la alimentación y la promoción de la vigilia. Sin embargo, actualmente se sabe que también participan en otros procesos comportamentales y fisiológicos, especialmente bajo circunstancias altamente motivacionales o emocionales, tales como la presencia de recompensas o la exposición a ciertas amenazas. Crecientes hallazgos indican que las orexinas podrían estar involucradas en un procesamiento inadecuado de estas situaciones, destacando su posible contribución en ciertos trastornos neuropsiquiátricos como la adicción a drogas y los trastornos de ansiedad. Mediante el uso de estudios comportamentales y bioquímicos, la presente tesis analiza el papel de las orexinas en los efectos farmacológicos y reforzantes de los cannabinoides, los cuales representan factores relevantes en la adicción a cannabis. Se observó que las orexinas contribuyen a los efectos hipotérmicos, antinociceptivos y ansiolíticos del Δ^9-tetrahidrocannabinol a través del receptor-2 de orexina, mientras que los efectos reforzantes del cannabinoide sintético WIN55,212-2 son modulados a través del receptor-1 de orexina, en parte mediante un mecanismo dependiente de dopamina. Por otro lado, investigamos la influencia de las orexinas en el procesamiento de recuerdos aversivos, un componente fundamental en diversos trastornos de ansiedad. Se confirmó el papel de ambos receptores de orexina en la consolidación del recuerdo del miedo, y se estableció que las orexinas, a través del receptor-1 de orexina, impiden una extinción adecuada de los recuerdos aversivos, debido posiblemente a la modulación de la comunicación entre la amígdala y la corteza prefrontal. Los hallazgos de la presente tesis revelan el potencial terapéutico del bloqueo del receptor-1 de orexina como nueva diana para prevenir la adicción a cannabis, así como para aliviar la retención anormal del miedo presente en trastornos de ansiedad tales como el trastorno de estrés postraumático y las fobias.

Abbreviations

2-AG	2-arachidonoylglycerol
BNST	bed nucleus of stria terminalis
BLA	basolateral amygdala
CB1R	cannabinoid receptor type 1
CB2R	cannabinoid receptor type 2
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CRF	corticotrophin-releasing factor
CS	conditioned stimulus
CSF	cerebrospinal fluid
DAGL	diacylglycerol lipase
DMH	dorsomedial hypothalamus
DORA	dual orexin receptor antagonist
DSM	Diagnostic and Statistical Manual of Mental Disorders
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amine hydrolase
GABA	γ-aminobutyric acid
GPCR	G protein-coupled receptor
HPA	hypothalamic-pituitary-adrenal
IP3	inositol trisphosphate
LC	locus coeruleus
LH	lateral hypothalamus
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase

MCH	melanin-concentrating hormone
NAc	nucleus accumbens
OX1R	orexin receptor type 1
OX2R	orexin receptor type 2
PFA	perifornical hypothalamic area
PFC	prefrontal cortex
PKC	protein kinase C
PLC	phospholipase C
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus of hypothalamus
PVT	paraventricular nucleus of thalamus
REM	rapid-eye movement
THC	Δ^9-tetrahydrocannabinol
US	unconditioned stimulus
VTA	ventral tegmental area

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INTRODUCTION

1. The orexin/hypocretin system

The discovery of the orexin/hypocretin system was simultaneously reported in 1998 by two independent groups through different scientific approaches in USA (de Lecea *et al*, 1998) and Japan (Sakurai *et al*, 1998). De Lecea and co-workers showed that a certain hypothalamic mRNA species encoded a potential secretory pro-peptide that gave rise to two peptide transmitters (de Lecea *et al*, 1998). One of these peptides was shown to be strongly neuroexcitatory in neuronal cultures. They named the peptides hypocretin-1 and hypocretin-2 ("hypo" for hypothalamus, "cretin" for the sequence resemblance to the hormone secretin). At the same time, the research group of Sakurai identified two peptide transmitters that activated an orphan receptor termed HFGAN72 (Sakurai *et al*, 1998). Subsequently, they identified a second receptor based on a sequence homology search, which responded to both peptides as well. These peptides were shown to derive from a common precursor peptide, and were able to stimulate food intake in rats upon intracerebro-ventricular infusion (Sakurai *et al*, 1998). The peptides were termed orexin-A and orexin-B (from *orexis*, the Greek word for appetite) and those no-longer-orphan receptors became the OX1R and OX2R orexin receptors. It soon became clear that hypocretin-1 and orexin-A as well as hypocretin-2 and orexin-B were the same peptides. Both sets of names are still in use.

These findings precipitated the emergence of an extensive amount of evidence describing the orexinergic neuronal circuits and cellular signalling pathways, and deciphering the function of this endogenous system in physiological and pathological conditions, which so far has resulted in more than 4.000 articles.

1.1. Overview of the orexin system

1.1.1. Orexin peptides

Orexin-A/hypocretin-1 (33 amino acids) and orexin-B/hypocretin-2 (28 amino acids) are two endogenous neuropeptides proteolytically cleaved from the same precursor peptide, prepro-orexin/prepro-hypocretin (131 amino acids) (de Lecea *et al*, 1998; Sakurai *et al*, 1998) (Figure 1). The

prepro-orexin gene encodes a single copy of each peptide and is located on chromosome 17 in humans (Sakurai *et al*, 1999). Orexin peptides are already present in fish (Alvarez and Sutcliffe, 2002), and are well conserved across mammalian species. The sequence of orexin-A is identical in rats, mice, pigs, dogs, and humans, whereas orexin-B differs only in one or two amino acids between these species (Wong *et al*, 2011). The strong preservation of the orexin system across vertebrate evolution reveals its functional relevance.

Diverse post-translational modifications take place in order to obtain the mature functional orexin peptides. Thus, both peptides are amidated in their COOH terminus. Orexin-A, in addition, is further stabilized with two intrachain disulphide bridges and its NH2-terminal glutamine is cyclized into a pyroglutamil residue (Sakurai *et al*, 1998). Orexin-A and orexin-B share 46% of their sequence (Sakurai *et al*, 1998), and their overall 3D structures are quite similar: they are both constructed of two α-helices at a 70º angle, explaining their ability to bind the same receptors (Kim *et al*, 2004; Lee *et al*, 1999). However, orexin-A appears to be more stable and lipophilic than orexin-B, a fact that might explain its ability to penetrate the blood-brain barrier under certain conditions (Kastin and Akerstrom, 1999). In the central nervous system (CNS), orexin peptides act as neuromodulators. Hence, they are stored in secretory vesicles, transferred through the axon to the neuronal terminals and released in a Ca^{2+}-dependent manner (de Lecea *et al*, 1998).

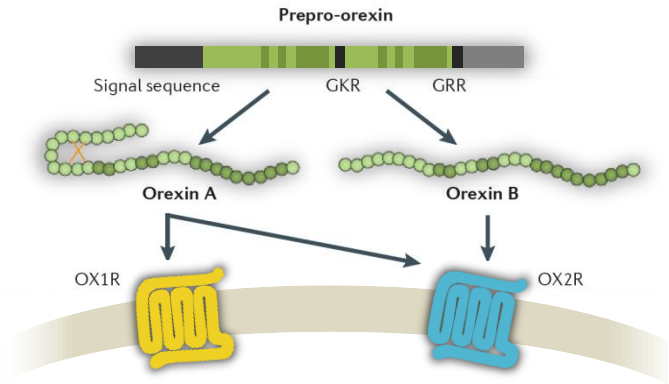


Figure 1. Orexin peptides and their receptors. Orexin-A and orexin-B are cleaved from their precursor prepro-orexin. Orexin-A contains two disulphide bridges. Orexins share 46% sequence identity (dark green shaded) and bind to OX1R and OX2R with different affinity. (Adapted from Sakurai, 2014).

1.1.2. Orexin receptors

Two receptors responsive to orexin stimulation have been identified so far: orexin/hypocretin receptor-1 (OX1R/Hcrtr-1, 425 amino acids) and orexin/hypocretin receptor-2 (OX2R/Hcrtr-2, 444 amino acids) (Figure 1) (Sakurai *et al*, 1998). Orexin receptors belong to the G protein-coupled receptor (GPCR) superfamily, specifically to the rhodopsin family. OX1R and OX2R genes are localized at chromosomes 1 and 6 in humans, respectively, and there is an overall 64% sequence identity between them (Kukkonen *et al*, 2002). Similar to their peptide counterparts, the sequence of orexin receptors is highly conserved across mammalian species. Thus, the cloned variants from mouse, rat, dog, pig and human share 91-98% sequence identity (Kukkonen *et al*, 2002). Although OX2R is present in most vertebrate lineages, OX1R-like genes have not been identified in non-mammalian species, suggesting that OX2R is likely to be evolutionary more ancient and OX1R evolved by gene duplication after the divergence that gave rise to mammals (Wong *et al*, 2011). Studies on heterologous expression systems have shown that orexin receptors differ in their ligand binding affinities. Thus, OX2R presents a rather equal affinity for both orexin peptides, while OX1R shows a 10- to 100-fold higher affinity for orexin-A than orexin-B (Ammoun *et al*, 2003; Sakurai *et al*, 1998). Nevertheless, binding affinities greatly vary depending on the expression system (Putula *et al*, 2011). It has been suggested that differences in the amino-terminal region of the orexin peptides might contribute for the high selectivity of OX1R to orexin-A, since the carboxi-terminal region is more conserved (Takai *et al*, 2006). Other neuropeptides have not been shown to bind to orexin receptors (Holmqvist *et al*, 2001).

1.1.3. Cellular signalling through orexin receptors

The signalling pathways triggered upon orexin receptor stimulation have been extensively investigated in transfected heterologous cell systems. However, these studies provide limited information about the particular signalling pathways taking place in native receptor-expressing neurons, from which available data are still limited (Kukkonen, 2013). It is commonly accepted that OX1R couple to Gq proteins and that OX2R couple to Gq and Gi/o family members. Nevertheless, it is often difficult to

determine G protein coupling of a GPCR due to the difficulty of direct measurements and the need to disrupt the cellular structure (Kukkonen, 2004). The G protein coupling of orexin receptors is far from clear, but based on the evidence available, both OX1R and OX2R can couple to Gq, Gs, and Gi (Bernard *et al*, 2003; Kukkonen and Leonard, 2014; van den Top *et al*, 2003; Yang *et al*, 2003). The main cellular pathways activated upon orexin receptor stimulation are represented in Figure 2.

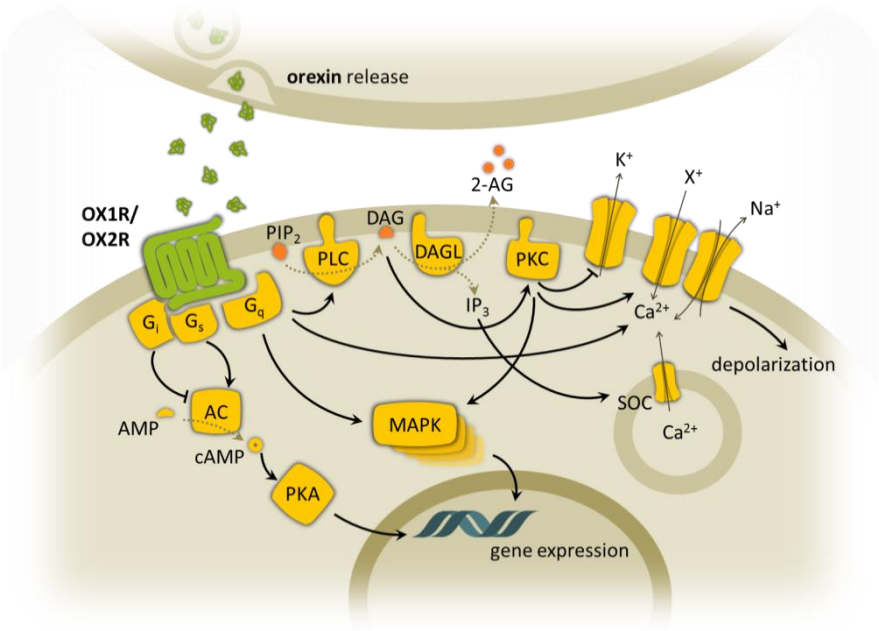


Figure 2. Main cellular signalling pathways activated upon orexin receptor stimulation. Orexin receptor stimulation is associated with Gq-dependent activation of the PLC/PKC pathway and diverse MAPK cascades, as well as membrane depolarization through modulation of cation channels. Gs and Gi protein stimulation has also been observed, leading to increase or decrease of AC activity, respectively. 2-AG, 2-arachidonoylglycerol; AC, adenylyl cyclase; DAGL, diacylglycerol lipase; IP3, inositol trisphosphate; MAPK, diverse members of the mitogen-activated protein kinase cascade; PIP2, phosphatidylinositol bisphosphate; PKA and PKC, protein kinases A and C; PLC, phospholipase C; SOC, store-operated Ca^{2+} channels.

Intracellular calcium in orexin receptor signalling

In neurons, the most frequent response after agonist binding to orexin receptors is an enhancement of intracellular Ca^{2+} concentrations, explaining the commonly reported neuroexcitatory nature of orexin peptides on the brain (Eriksson *et al*, 2001; van den Pol *et al*, 1998). The

transduction mechanism responsible for increasing intracellular Ca^{2+} levels upon orexin receptor activation is based on the activation of Gq proteins, which induces the stimulation of phospholipase C (PLC) and subsequent production of the second messengers diacylglycerol and inositol trisphosphate (IP3) from membrane phospholipids. This triggers the activation of protein kinase C (PKC), which phosphorylates and modulates effector ion channels leading to Ca^{2+} entrance (Kohlmeier *et al*, 2004; Uramura *et al*, 2001; Xia *et al*, 2009), as well as further IP3-mediated entry via store-operated Ca^{2+} channels (Kukkonen and Akerman, 2001; Larsson *et al*, 2005). Orexin receptor activation can also lead to an increase in the intracellular Ca^{2+} concentration and membrane depolarization by PLC-independent mechanisms that include an increase of non-selective cation channel conductances (Liu *et al*, 2002; Murai and Akaike, 2005; Yang and Ferguson, 2002), activation of the Na^{+}/Ca^{2+} exchanger (Burdakov *et al*, 2003) and/or blockade of inward-rectifying K^{+} channels (Hwang *et al*, 2001; Ishibashi *et al*, 2005; Yang and Ferguson, 2003). Nevertheless, orexins have also been reported to reduce intracellular Ca^{2+} levels through Gi protein activation upon OX2R stimulation in proopiomelanocortin neurons (Muroya *et al*, 2004).

Adenylyl cyclases as effectors of orexin signalling

Activation of adenylyl cyclase leads to the production of cAMP and activation of protein kinase A and cAMP-regulated ion channels (Patel *et al*, 2001). Stimulation of both orexin receptors has been shown to activate adenylyl cyclase and cAMP production not only in recombinant cell lines (Holmqvist *et al*, 2005; Tang *et al*, 2008), but also in neurons (Gorojankina *et al*, 2007; van den Top *et al*, 2003) and astrocytes (Woldan-Tambor *et al*, 2011). In addition, OX1R is also able to inhibit adenylyl cyclase via Gi coupling (Holmqvist *et al*, 2005; Kukkonen, 2016; Urbańska *et al*, 2012).

Activation of protein kinases

As mentioned above, the activation of PLC upon OX1R stimulation leads to the production of diacylglycerol and concomitant activation of PKC. Among other effectors, PKC phosphorylates extracellular signal-regulated kinase (ERK) and p38 kinase, both in recombinant cells (Ammoun *et al*, 2006a; Tang *et al*, 2008) and in neurons (Gorojankina *et al*, 2007; Selbach

et al, 2010; Shin *et al*, 2009). These kinases are two well-known members of the mitogen-activated protein kinase (MAPK) pathway, which is involved in several cellular processes, including synaptic plasticity, cell survival and proliferation (Selbach *et al*, 2010; Thornton and Rincon, 2009). Similar results have been recorded in an OX2R expression system (Guo and Feng, 2012; Tang *et al*, 2008). In addition to the PLC/PKC pathway, other downstream effectors contribute to ERK1/2 activation after orexin receptor stimulation, including at least Ca^{2+} influx, Ras, Src, and phosphatidylinositide 3-kinases (Ammoun *et al*, 2006b).

Lipid mediators of orexin signalling

Besides the activation of PLC and subsequent production of the lipid messengers diacylglycerol and IP3, orexin receptor stimulation also leads to activation of phospholipases A2 and D (Jäntti *et al*, 2012; Turunen *et al*, 2010). Phospholipase A2 gives rise to arachidonic acid release, a lipid signal that can regulate the activity of different cation channels generating oscillatory Ca^{2+} signals (Meves, 2008; Shuttleworth, 2009). Orexin-induced arachidonic acid release seems to be a relevant response since it already occurs at very low agonist concentrations (Shuttleworth, 2009). Active phospholipase D hydrolyses phosphatidylcholine to produce phosphatidic acid (Jäntti *et al*, 2012), which in turn activates several kinases involved in cellular proliferation, vesicle trafficking, and cell migration (Jang *et al*, 2012). Finally, orexin receptor activation has been reported to release the endocannabinoid 2-arachidonoylglycerol (2-AG) via the activation of PLC and diacylglycerol lipase (DAGL) (Haj-Dahmane and Shen, 2005) *(For further details see section: 2.3.8. Cross-talk between orexin and endocannabinoid systems).*

1.2. Neuroanatomical distribution of the orexin system

In the CNS, orexin-expressing neurons constitute a relatively small population of cells located in certain subregions of the hypothalamus, including the lateral hypothalamus (LH), the perifornical area (PFA), and the dorsomedial hypothalamus (DMH) (Figure 3A) (de Lecea *et al*, 1998; Peyron *et al*, 1998; Sakurai *et al*, 1998). It has been estimated that there are barely 3.000 orexinergic neurons in the rat brain (Nambu *et al*, 1999) and 70.000 in the human brain (Sakurai and Mieda, 2011). They do not

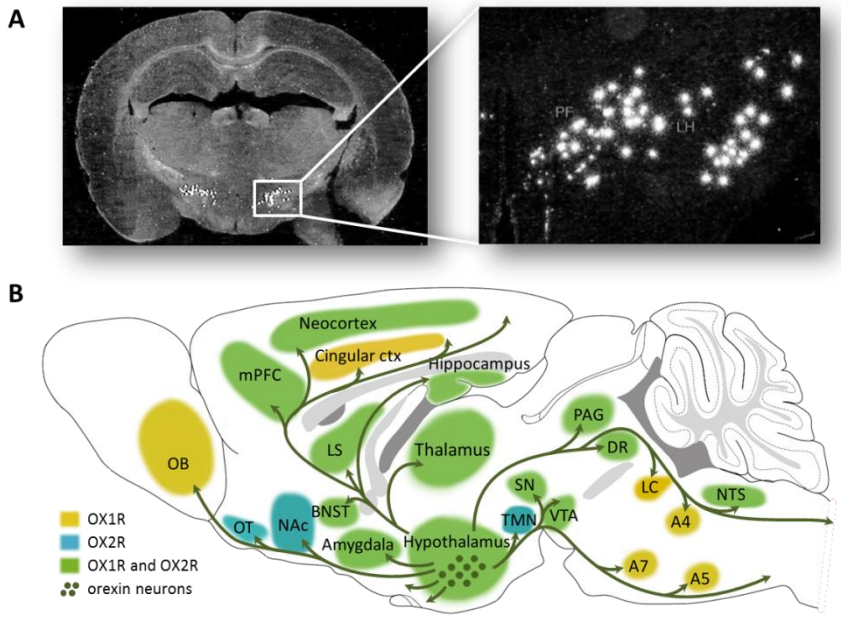


Figure 3. Localization of orexin-expressing neurons and distribution of orexin receptors. A. *In situ* hybridization with a ^{33}P-labelled antisense riboprobe for *orexin* exon 2 in a coronal section of the rat brain. The signal is exclusively localized in the lateral hypothalamic area. **B.** Organization of the orexin neuronal system in a sagittal section of mouse brain. Orexin neurons send axonal projections to many brain areas (arrows). Orexin receptors show partially overlapping expression patterns. A4, A5, A7, pons cell groups; ctx, cortex; DR, dorsal raphe ; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; SN, substantia nigra; TMN, tuberomammillary nucleus; VTA, ventral tegmental area. (Adapted from Chemelli *et al*, 1999, and Kukkonen, 2013).

form a distinct nucleus but are mixed with other neurons, such as melanin-concentrating hormone (MCH)-expressing neurons (Broberger *et al*, 1998; Peyron *et al*, 1998). Apart from orexin peptides, orexinergic neurons express a multitude of other transmitters and signalling molecules. These include the opioid peptide dynorphin (Bayer *et al*, 2002; Chou *et al*, 2001), galanin (Håkansson *et al*, 1999), prolactin (Risold *et al*, 1999), and neuronal activity-regulated pentraxin (Crocker *et al*, 2005; Reti *et al*, 2002). Furthermore, particular subpopulations of orexin cells have been reported to release glutamate at the synaptic terminal (Henny *et al*, 2010; Rosin *et al*, 2003; Schöne *et al*, 2012; Torrealba *et al*, 2003). The role of these other putative transmitters in the functions of orexinergic neurons remains rather unclear (Schöne and Burdakov, 2012).

Despite small in number, orexin-containing neurons project widely throughout the entire CNS, suggesting that hypocretins modulate the activity of multiple neurotransmitter systems and regulate diverse physiological functions (Peyron *et al*, 1998) (Figure 3B). The most important orexinergic projection areas are within the hypothalamus, denoting an important function of this system in energy homeostasis and central regulation of neuroendocrine and autonomic functions (Sakurai, 2006). The most significant extra-hypothalamic projections are found within diverse brainstem nuclei related with the regulation of anxiety-like responses and sleep/wake cycles, including the raphe nuclei, the reticular formation, and especially the locus coeruleus (LC) (Peyron *et al*, 1998), in agreement with the role of these neuromodulators in the maintenance of the waking-state and arousal promotion (Eriksson *et al*, 2010). Orexin neurons also send substantial efferent projections to diverse structures of the mesocorticolimbic system, such as the ventral tegmental area (VTA), the septal nuclei, the bed nucleus of stria terminalis (BNST), and the amygdaloid nuclei, supporting the regulation of natural reward and addiction processes by orexins. Other areas within the reward circuitry, such as the shell of the nucleus accumbens (NAc) and the prefrontal cortex (PFC), also receive disperse orexinergic innervation (Baldo *et al*, 2003; Peyron *et al*, 1998; Plaza-Zabala *et al*, 2012b).

The expression of orexin receptors has been investigated mainly in rats by determining OX1R and OX2R mRNA levels (Marcus *et al*, 2001; Trivedi *et al*, 1998), and is convergent with the presence of orexin fibres. Although the expression patterns of OX1R and OX2R are partially overlapping, certain areas particularly express one receptor subtype, suggesting that OX1R and OX2R may have different physiological roles. Thus, the PFC, the hippocampal CA1 and CA2 areas, and the LC predominantly express OX1R (Marcus *et al*, 2001). Conversely, the NAc and the tuberomammillary nucleus mainly express OX2R (Marcus *et al*, 2001; Trivedi *et al*, 1998).

Growing evidence supports the presence of orexin peptides and receptors also in tissues outside of the CNS, such as the gastrointestinal tract, pancreas, adrenal gland, kidney, adipose tissue and reproductive glands (Heinonen *et al*, 2008; Kirchgessner, 2002). This distribution suggests that orexins might modulate diverse endocrine functions and energy balance processes through peripheral actions. However, the sources and functions

of orexins in peripheral tissue are not completely understood and are beyond the scope of this thesis, which rather studies the central functions of the orexin system.

1.3. Physiological functions in the CNS

Orexinergic fibres and orexin receptors distributed throughout the entire CNS are in anatomical correspondence with the variety of physiological functions of the orexin system, including regulation of arousal and sleep/wake cycles, appetite and energy homeostasis, reward-seeking and reinforcement, and stress response and anxiety, among others (Sakurai and Mieda, 2011). Accordingly, dysfunction of the orexin system contributes to some neuropsychiatric disorders where these physiological aspects are particularly compromised, such as narcolepsy, eating disorders, drug addiction, and anxiety disorders (Chen *et al*, 2015). Recent evidence also points to a role for the orexin system in other CNS disorders, such as Alzheimer's disease (Fronczek *et al*, 2012).

1.3.1. Arousal, wakefulness and sleep

Regulation of sleep/wakefulness is the best understood role of the orexin system. Extensive research has evidenced that orexins are both sufficient and necessary for maintaining the waking state, and they are now generally considered to be arousal-promoting peptides (Sakurai, 2007; Saper *et al*, 2005). Indeed, orexin neurons display high firing rates during the waking period and cease their discharge during both rapid-eye movement (REM) and non-REM sleep (Lee *et al*, 2005). Orexin neurons are also connected to a multitude of nuclei at different levels of CNS involved in sleep/wake cycle (Alexandre *et al*, 2013). Thus, orexins activate the neurons in the dorsal raphe (Brown *et al*, 2001), tuberomammillary nucleus (Bayer *et al*, 2001; Eriksson *et al*, 2001), and LC (Hagan *et al*, 1999; Horvath *et al*, 1999), key regions in the maintenance of wakefulness and arousal. In turn, it has been suggested that orexin neurons are inhibited during sleep by a population of γ-aminobutyric acid (GABA)ergic neurons projecting from the ventrolateral preoptic area, a sleep-promoting region (Sakurai *et al*, 2005; Tsujino and Sakurai, 2013). Accordingly, central administration of orexin-A or -B in rodents during the light (rest) cycle increases the awake time and decreases both REM and

non-REM sleep episode number and duration (Bourgin *et al*, 2000; España *et al*, 2001; Piper *et al*, 2000). Optogenetic activation of orexin neurons similarly increases the probability of an awakening event during sleep phases (Adamantidis *et al*, 2007; Carter *et al*, 2009), whereas silencing these neurons induces slow-wave sleep in mice (Tsunematsu *et al*, 2011). The tuberomammillary-histaminergic pathway might be an important effector site of orexin for sleep/wake regulation, since these effects of orexin-A are blocked by histamine H1 receptor antagonists or gene disruption in mouse (Huang *et al*, 2001; Yamanaka *et al*, 2002).

Narcolepsy, a chronic neurological disorder characterized by daytime sleepiness, cataplexy, and striking transitions from wakefulness into REM sleep, constitutes the clearest evidence of orexin relevance in sleep/wakefulness pathophysiology. Narcoleptic patients show an 80-100% reduction in the number of neurons containing detectable prepro-orexin mRNA and orexin-like immunoreactivity (Peyron *et al*, 2000; Thannickal *et al*, 2000), as well as reduced orexin-A levels in their cerebrospinal fluid (CSF) (Nishino *et al*, 2000). Therefore, a low orexin-A CSF level is now one of the diagnostic criteria for narcolepsy–cataplexy, according to the international classification of sleep disorders (American Academy of Sleep Medicine, 2005). Findings from animal models have further supported the conception of narcolepsy as a dysfunction of orexinergic neurons. Mice lacking the prepro-orexin gene (Chemelli *et al*, 1999), genetic ablation of orexinergic cells (orexin/ataxin-3 transgenic) (Hara *et al*, 2001) and pharmacological destruction of orexinergic neurons (Gerashchenko *et al*, 2001) result in phenotypes strongly parallel to the human condition, characterized by cataplexy-like attacks, occasional direct transitions to REM sleep from wakefulness, and highly fragmented sleep-wake cycles. Dogs with an inactivating mutation of the OX2R gene also show a phenotype remarkably similar to human narcoleptic patients (Lin *et al*, 1999). Accordingly, OX2R knockout mice show also a narcoleptic-like phenotype (Willie *et al*, 2003), whereas OX1R knockout mice exhibit only mild fragmented sleep-wake cycle (Jon T. Willie *et al*, 2003). However, double OX1R/OX2R knockout mice, but not single OX2R knockouts, are affected by with cataplexy-like attacks of REM sleep (Willie *et al*, 2003). Therefore, the current view is that although OX2R receptor

has a pivotal role in the promotion of wakefulness, OX1R is also required for an accurate modulation of the sleep cycle (Tsujino and Sakurai, 2013).

1.3.2. Feeding behaviour and metabolism

Orexins were initially reported to be regulators of feeding behaviour based on their capacity to elicit food intake when centrally administered to rats (Sakurai *et al*, 1998). Many studies have subsequently replicated this effect in mice, and even in zebrafish (Haynes *et al*, 2000; Thorpe and Kotz, 2005; Yokobori *et al*, 2011). Conversely, intraventricular administration of an anti-orexin antibody or an OX1R antagonist, as well as genetic ablation of orexin neurons, attenuates food consumption (Hara *et al*, 2001; Haynes *et al*, 2000; Yamada *et al*, 2000). One of the mechanisms by which orexins induce food intake is the activation of neurons in arcuate nucleus expressing neuropeptide Y, a peptide known for its orexigenic effects (Dube *et al*, 2000; Jain *et al*, 2000; Yamanaka *et al*, 2000).

Orexin neurons are able to monitor humoral and neural indicators of energy balance. High extracellular levels of glucose and leptin, an hormone from adipose tissue reducing food intake, inhibit the activity of orexin neurons (Burdakov *et al*, 2005; Yamanaka *et al*, 2003). Conversely, decreased concentrations of glucose or ghrelin, a fasting-induced hormone secreted by the stomach, activate the orexinergic cells (Briski and Sylvester, 2001; Otlivanchik *et al*, 2015; Toshinai *et al*, 2003). In line with these results, food deprivation induces the expression of prepro-orexin in the hypothalamus, as well as OX1R and OX2R mRNA levels in diverse brain regions (Cai *et al*, 1999; Lu *et al*, 2000; Sakurai *et al*, 1998). These findings suggest that orexin neurons monitor indicators of energy balance of the body and mediate adaptive augmentation of arousal, and in turn of feeding behaviour, in response to fasting. Interestingly, orexin-neuron ablated mice, despite exhibiting hypophagia, display an obese phenotype (Hara *et al*, 2001), and narcoleptic humans have increased body mass index although their caloric intake is lower (Lammers *et al*, 1996; Schuld *et al*, 2000). A possible explanation for these observations emerges from studies indicating that orexins contribute also to increase body energy expenditure (Lubkin and Stricker-Krongrad, 1998; Wang *et al*, 2001). Thus, central administration of orexin-A has been reported to

increase energy consumption by increasing spontaneous physical activity and thermogenesis (Kotz *et al*, 2002; Yoshimichi *et al*, 2001). Moreover, orexin overexpressing mice have been reported to be resistant to diet-induced obesity (Funato *et al*, 2009). Therefore, orexin signalling might positively regulate feeding and arousal, but also motor activity and basal energy expenditure, resulting in resistance to weight gain.

1.3.3. Reward

The orexin system is also involved in the regulation of the mesocorticolimbic rewarding system, a circuit responsible for the pleasurable feelings associated with natural rewards and the consumption of drugs of abuse. The main components of this reward circuit are the VTA, which contains the dopaminergic cell bodies, and its target areas, including the NAc, amygdala, and frontal and limbic cortices (Wise, 2004) *(For further details see section: 2.1. Neurobiology of drug addiction)*. Immunohistochemical studies indicated a dense projection of orexin fibres from LH to the reward system (Peyron *et al*, 1998), as well as orexin receptor mRNA distribution in these areas (Marcus *et al*, 2001; Trivedi *et al*, 1998). The dopamine neurons in the VTA might be a crucial site of action for orexins to mediate these effects, since intra-VTA infusion of orexin-A and -B increased dopamine release in NAc and PFC as measured by *in vivo* microdialysis and voltammetry (España *et al*, 2011; Vittoz *et al*, 2008). Orexins elicit their influence on VTA dopamine cell firing not only via direct depolarization of dopamine neurons (Korotkova *et al*, 2003), but also interacting with other neurotransmitters within the VTA, such as glutamate (Borgland *et al*, 2006). The modulation of the reward circuit by the orexin system seems to influence also feeding behaviour. Orexins promote food consumption not only as a response to variations in the nutritional status and energy stores, but also because they modulate the cognitive component influenced by the hedonic aspects of eating (Saper *et al*, 2002). Thus, OX1R antagonism not only alters operant seeking behaviour for standard food in food-restricted mice (Sharf *et al*, 2010b), but also reduces both motivational and primary reinforcement in rats trained to seek for high-palatable food, even under satiation (Choi *et al*, 2010). Similarly, orexins modulate the addictive properties of several drugs of abuse, such as alcohol, nicotine, opioids, and psychostimulants

(Plaza-Zabala *et al*, 2012b). A summary of evidences supporting the role of the orexin system in addiction is further discussed in section *2.2. Role of the orexin system in drug reward and addiction.*

1.3.4. Stress and anxiety

Stress is a natural reaction of the body to an internal or external challenge in order to prepare a physiological and behavioural response that enables dealing with the stressor stimulus. Several findings support the idea that the orexin system contributes to respond to acute stress (Winsky-Sommerer *et al*, 2005). Indeed, orexinergic neurons, especially those located in the PFA/DMH, are activated by different stressors, including immobilization, footshock, cold exposure, and food deprivation (Berridge *et al*, 2010; Johnson *et al*, 2012a). Consistent with this, intracerebroventricular administration of orexin-A induces anxiety-like effects in diverse behavioural models of anxiety (Suzuki *et al*, 2005) and stimulates the hypothalamic-pituitary-adrenal (HPA) axis (Kuru *et al*, 2000). Moreover, central infusion of orexin activates neurons expressing corticotrophin-releasing factor (CRF) in the paraventricular nucleus of the hypothalamus (PVN) and the central amygdala (Sakamoto *et al*, 2004). Direct reciprocal interactions have been described between orexin neurons and CRF-expressing neurons of the PVN, suggesting a cross-modulation between the two systems (Winsky-Sommerer *et al*, 2004). Thus, application of CRF to hypothalamic slices depolarizes membrane potential and increases firing rate in a subpopulation of orexin-expressing cells (Winsky-Sommerer *et al*, 2004). In turn, application of orexin-A depolarizes and increases spike frequency in magnocellular and parvocellular neurons of the PVN (Samson *et al*, 2002). This suggests that the orexin system is an important contributor to the physiological CRF-mediated behaviours that occur in response to stressful situations (Giardino and de Lecea, 2014). Dysregulation of stress responses can promote the development of different anxiety disorders, which might be influenced by the activity of the orexin system. Further discussion regarding the contribution of orexins to anxiety disorders, particularly those presenting maladaptive fear, is included in section *3.3. Role of orexins in fear and anxiety.*

In summary, orexins modulate a wide variety of physiological processes, and more importantly, dysfunction of the orexin system contributes to diverse neuropsychiatric disorders. Although a considerable amount of research has already been performed in the orexin field, many aspects of their increasingly important role in neuropsychiatry remain unknown. This thesis is mainly focused in two of these unexplored aspects: (1) the role of orexins in cannabinoid addiction, and (2) the role of orexins in fear memory processing, a pivotal component of some anxiety disorders. Since the most feasible therapeutic strategies targeting the orexin system are currently based on orexin receptor pharmacological manipulation, the use of orexin receptor ligands has constituted our main research tool, complemented in some cases with studies in transgenic mice.

1.4. Orexin receptor ligands

Soon after the discovery of orexins, the need for pharmacological tools to manipulate this endogenous neuromodulator system motivated several pharmaceutical companies to develop synthetic orexin receptor ligands. This research was developed to allow further biomedical research in the unexplored field of orexins, but also as potential therapeutic agents for medical conditions related with orexin dysfunction. Table 1 displays the chemical structure and selectivity profiles of the main orexin receptor ligands, all of them antagonists.

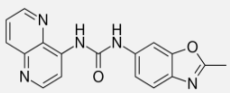
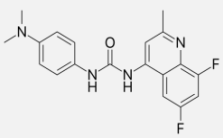
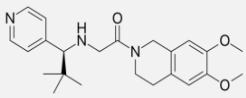
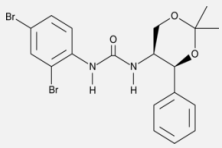
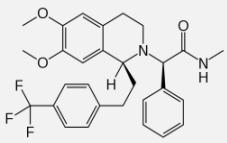
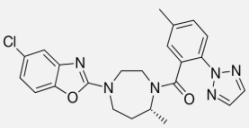
1.4.1. Orexin receptor ligands as research tools

Orexin receptor antagonists

The first orexin receptor antagonist, SB-334867, was developed by GlaxoSmithKline soon after the discovery of orexins (Smart *et al*, 2001). Its affinity for OX1R is 50-fold higher than for OX2R, although *in vivo* studies using high doses (>20 mg/kg) should be interpreted cautiously because those doses may block both receptors (Scammell and Winrow, 2011). It has been extensively used in orexin research due to its favourable preclinical pharmacokinetics, such as rapid absorption and brain penetration following intraperitoneal administration (Porter *et al*, 2001). In addition, GlaxoSmithKline has developed other selective OX1R antagonists, including SB-408124 and SB-674042, also used, to a smaller

extent, in biomedical research (Ibrahim and Abdel-Rahman, 2015; Jeon *et al*, 2015; Langmead *et al*, 2004).

Table 1. Representative list of orexin receptor antagonists. (Data from Lebold *et al*, 2013, and Xu *et al*, 2013). na, not available data.

Compound	Structure	Selectivity	pKi (nM)	
			OX1R	OX2R
SB-334867		OX1R 50x OX2R	28	1704
SB-408124		OX1R 64x OX2R	22	1405
TCS-OX2-29		OX2R 250x OX1R	na	7.5
JNJ-10397049		OX2R 630x OX1R	1644	6
ACT-078573 (almorexant)		OX2R 1.6x OX1R	13	8
MK-4305 (suvorexant)		OX2R 0.9x OX1R	0.6	0.4

A research team at Banyu Pharmaceuticals developed the first OX2R selective antagonist, TCS-OX2-29 or "compound 29" (Hirose *et al*, 2003). It presents a >250-fold higher affinity for OX2R than for OX1R, and its high selectivity and water solubility constitute its main benefits (Boss *et al*, 2009). Researchers at Johnson & Johnson described a series of compounds with around 700-fold selectivity for antagonizing OX2R (McAtee *et al*, 2004), from which the OX2R antagonist JNJ-10397049 is purchasable for research (Dugovic *et al*, 2009). Another potent OX2R antagonist, known as EMPA, has been developed by Hoffmann–La Roche. This ligand exhibits >900-fold selectivity in binding to OX2R over OX1R (Malherbe *et al*, 2009a). Since the commercial release of OX2R antagonists was considerably posterior than for OX1R antagonists, relatively few articles have been published by using these tools, particularly in animal models. Results from ongoing research regarding OX2R function will be surely revealed during the ensuing years.

A series of dual orexin receptor antagonists (DORAs) have also been developed during the last ten years. Most of them were synthetized and subjected to preclinical assessment, prior to undergo clinical evaluation for treating insomnia (see below), but application of these compounds in biomedical investigation has been modest (Scammell and Winrow, 2011). Almorexant (ACT-078573), a potent DORA described by Actelion in 2007, is the main dual antagonist employed in orexin research (Brisbare-Roch *et al*, 2007). Almorexant appears to be a competitive antagonist of OX1R and a non-competitive antagonist of OX2R (Malherbe *et al*, 2009b). It penetrates the brain well, and has a good absorption in rats and dogs even after oral administration (Brisbare-Roch *et al*, 2008). This ligand has been useful in research, but their favourable pharmacology and strong sleep-promoting effects in animals launched almorexant to evaluation in clinical trials.

Orexin receptor agonists

The design of small-molecule agonists of orexin receptors, and of all GPCRs in general, is considered as one of the current challenges in drug discovery. Such ligands hold high therapeutic potential and would provide considerable benefits as good tool compounds for exploration of orexin receptor function. Natural orexin neuropeptides are nonselective and are

often inefficient or troublesome for *in vivo* studies because of their poor ability to penetrate the blood–brain barrier. Due to the particular difficulty of mimicking the effects of these large peptides with small molecules, natural orexin peptides are the main available orexin agonists (Kukkonen, 2013). A truncated orexin peptide-derivative with higher OX2R affinity than the native peptide, [Ala(11), D-Leu(15)]orexin-B, has also been described (Asahi *et al*, 2003) and employed, to a smaller extent, in biomedical research. Since OX2R is considered to be non-specific for orexin-A and -B but OX1R is thought to show 10- to 100-fold higher affinity for orexin-A, it has been assumed that using orexin-A and orexin-B to distinguish between OX1R and OX2R in native systems is a reasonable strategy (Kukkonen *et al*, 2002). However this distinction is not always valid considering that orexin-A and-B present similar potency to increase intracellular Ca^{2+} level through OX1R stimulation in determined conditions (Putula *et al*, 2011). Therefore, studies only using agonists to determine involvement of the orexin receptor subtypes must be interpreted carefully, particularly in native systems, where multiple types of receptors may contribute to the measured output (Kukkonen, 2013). Recently, the first non-peptidic orexin agonist, called "compound 26", has been synthetized (Nagahara *et al*, 2015). This synthetic compound is highly OX2R selective, and due to its chemical profile it is presumed to cross the blood-brain barrier, entailing obvious advantages as a research tool, but also for future possible medical application (Heifetz *et al*, 2015).

1.4.2. Orexin receptor ligands as therapeutic agents

As described above, extensive evidence suggests that the orexin system contributes to the pathophysiology of several human medical conditions. These findings have encouraged pharmaceutical companies to develop drugs targeting orexin receptors as novel therapeutic agents for sleep disorders, drug addiction, and mood and anxiety disorders, among others. So far diverse compounds –all of them DORAs– are under current or recent evaluation in clinical trials, predominantly focused on insomnia treatment. One of them, suvorexant, is already commercially available for therapeutic use.

Orexin receptor antagonists for treating insomnia

Due to the involvement of orexins in the maintenance of arousal, orexin receptor antagonists have been proposed as potential new drugs for the treatment of chronic insomnia, which is defined as difficulty initiating and/or maintaining sleep on at least three nights per week for at least three months (Ohayon, 2002). Currently, benzodiazepines are the most frequently prescribed drugs for treating this condition (Scammell and Winrow, 2011). Benzodiazepines are positive allosteric modulators of $GABA_A$ receptors and widely inhibit neuronal activity. Despite their efficacy, benzodiazepines usually present adverse effects, such as residual sedation, memory impairment, and abuse and physical dependence (Tan *et al*, 2010). Orexin receptor antagonists appear as new alternative strategy for these traditional therapies. The first proof-of-concept of this strategy was demonstrated with almorexant, which presented good tolerability and increased sleep efficiency in encouraging Phase I and II studies (Hoever *et al*, 2012). Unfortunately, Phase III studies were discontinued in 2011, probably due to the occurrence of adverse effects (Actelion, 2011). In contrast, suvorexant (MK-4305), another DORA developed by Merck, was recently approved by the US Food and Drug Administration for insomnia treatment in adults (available in the USA as Belsomra®). Suvorexant improved sleep onset and maintenance in Phase I and II clinical trials and was generally safe and well tolerated during the whole treatment period, although day somnolence appeared as common adverse effect (Herring *et al*, 2016; Michelson *et al*, 2014). Indeed, a recent FDA report raised concerns about the safety of suvorexant for treating insomnia mainly when used at high doses (Farkas, 2013) and recommended additional efforts to find its lowest effective dose. Three other dual antagonists are also now under development for insomnia treatment. ACT-462206 (Hoch *et al*, 2014) is under development in Phase I, and SB-649868 (Bettica *et al*, 2012) and filorexant (MK-6096) are both under development in Phase II.

Orexin receptor antagonists in other medical conditions

Novel clinical trials are also being performed to evaluate the possible effectiveness of DORAs in other pathologies. Recently, a Phase II trial reported that filorexant was effective in preventing migraine when

administered at night (Chabi *et al*, 2015). Similarly to suvorexant, the most common adverse effect in patients treated with filorexant was day somnolence. Filorexant is currently undergoing other clinical trials to evaluate its possible efficacy for treating neuropathic pain and major depressive disorder. The encouraging results obtained in these studies provide the basis to design novel clinical trials to evaluate the effectiveness of orexin antagonists for other medical conditions, including drug addiction and anxiety disorders.

Orexin receptor agonists as anti-narcoleptic drugs

Since narcolepsy is a condition characterized by orexin deficiency, replacement therapy using orexin receptor agonists has been proposed as potential therapeutic strategy for narcolepsy. Currently, daytime sleepiness is treated with psychostimulants, whereas cataplexy is treated with antidepressants (Mieda and Sakurai, 2013). Unfortunately, these therapeutic strategies present limited effectiveness, undesirable side effects, and drug abuse potential in the case of psychostimulants (Mieda and Sakurai, 2013). The potential effectiveness of orexin replacement therapy is further supported by a study in orexin neuron-ablated mice reporting effective reversion of narcoleptic-like phenotype after central infusion of orexin-A (Mieda *et al*, 2004). Nevertheless, development of small-molecule agonists is required to apply this therapeutic strategy in humans. The recent development of the first non-peptidic orexin agonist "compound 26" (Nagahara *et al*, 2015) brings us closer to the possibility of treating pathologies with deficient orexin signalling.

2. Addiction: focusing on cannabinoids

As mentioned, strong evidence supports that orexin transmission has a pivotal role in reward processing and subsequent impact on drug use and abuse. In this section, the neurobiological basis of drug addiction will be summarized first, followed by a description of the main findings supporting the participation of orexins in cocaine, opioids, alcohol and nicotine addiction. The possible role of the orexin system in the addictive properties of cannabis has not been evaluated so far, and exploring this particular aspect represents one of the main objectives of this thesis. Therefore, an outline of cannabis addiction and the effects of cannabinoid compounds will be additionally discussed.

2.1. Neurobiology of drug addiction

2.1.1. Addiction: a chronic brain disease

Drug addiction is a chronic brain disease characterized by compulsive drug seeking and drug taking despite possible harmful consequences, loss of control in limiting drug intake, emergence of a negative emotional state when drug access is prevented, and relapse to drug-seeking even after long periods of abstinence (Koob and Le Moal, 1997, 2008a). Drug addiction has been traditionally underestimated and it has not been always accepted as a disease with a neuropathological basis (Hyman, 2007). Although it has been considered to reflect “defects of character” that drive affected individuals to engage in “bad” behaviour, years of research have made it clear that addiction to drugs is based on pathological changes in brain function produced by repeated pharmacological insult to specific brain circuits. These circuits are involved in reward and learning processes that regulate how a person interprets and behaviourally responds to motivationally relevant stimuli (Kalivas and O’Brien, 2008). Hence, repeated stimulation of motivational circuitries by addictive drugs leads to maladaptive changes that progressively redirect the behavioural strategies, originally driven in response to biological stimuli, towards drug-seeking and drug-taking (Kalivas and O’Brien, 2008). Over the course of this progression, 3 stages of drug use have been differentiated: (1) the occasional, controlled or social use, in order to

obtain their rewarding and desired effects (2) drug abuse, where the method or the amount of the drug consumed is potentially harmful, and (3) drug addiction (Figure 4). Currently, one of the key purposes of neurobiological research is to elucidate the progressive neuroadaptive changes that contribute to the transition from controlled drug use to drug addiction (Koob and Volkow, 2010).

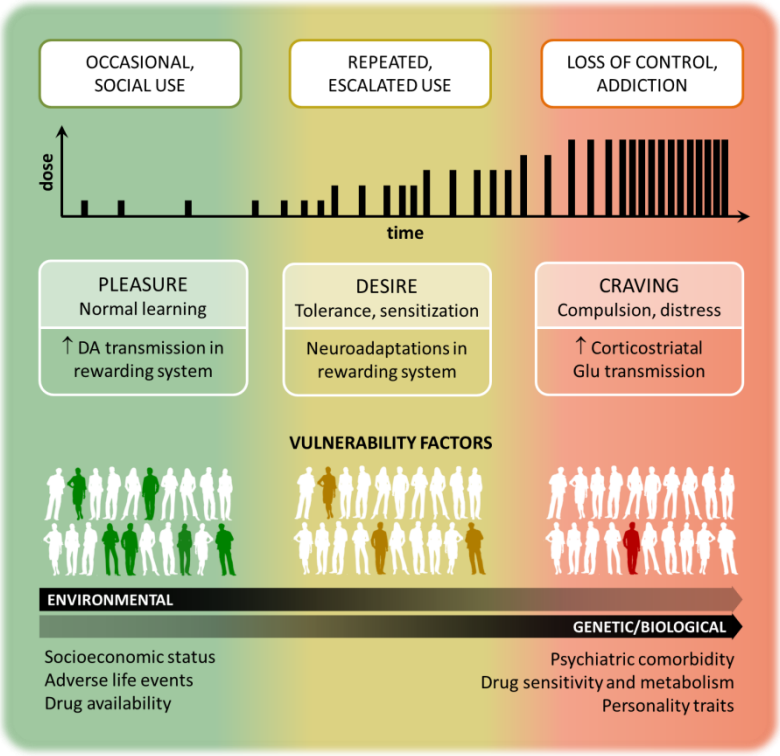


Figure 4. Stages of transition to addiction. Transition to addiction is a progression of three consecutive phases: (1) occasional, social drug use, in which drug intake is recreational and sporadic; (2) repeated, escalated drug use, in which drug intake intensifies frequency and intake amount; (3) loss of control and full drug addiction, where drug-devoted activities are the principal occupations of the individual. Specific individual vulnerabilities contribute to this progression. It is considered that environmental factors have a stronger effect on initiation, whereas biologic/genetic factors play a larger role in the transition from regular use to the development of addiction. The first phase occurs in a considerable number of individuals, who perceive drug exposure as extremely salient due to overstimulation of the rewarding system. During the second phase a series of plastic changes take place within the mesocorticolimbic system (such as a hyperactive –sensitized– dopaminergic system and an impaired prefrontal control) that makes drugs strongly wanted. In the last phase, increased corticostriatal transmission contributes to automatization of drug-taking and seeking, and drug absence is experienced as an irreplaceable loss. (Adapted from Kalivas and O'Brien, 2008, and Piazza and Deroche-Gamonet, 2013).

Drug addiction represents an important public health concern. Considered together, alcohol, tobacco, and illicit drugs are involved in over 12% of mortality worldwide, and their use constitutes the leading cause of preventable death (Swendsen and Le Moal, 2011). However, these rates are principally explained by a small portion of all substance users since only certain individuals of those who are exposed to drugs of abuse will develop a substance-related disorder. Thus, it is estimated that the percentage of consumers that develop addiction as a function of ever having tried a drug varies from approximately 9% for marijuana to 31% for tobacco (Anthony *et al*, 1994; Wagner and Anthony, 2002). An individual's probability of initial use and propensity of progression toward a pathologic pattern of use are influenced by a broad range of vulnerability factors (Figure 4) (Swendsen and Le Moal, 2011). Hence, drug addiction has a multi-factorial aetiology, and the interplay between multiple genetic and environmental variables strongly contributes to an individual's susceptibility or resilience to start drug consumption and to develop an addictive disorder (Ducci and Goldman, 2012; Nielsen *et al*, 2012). These vulnerability factors include intrinsic factors (e.g., personality traits, genetic factors, comorbidity with other psychopathological conditions) and extrinsic factors (e.g., socioeconomic status, adverse life events, drug availability). In addition, the potential for abuse also depends on the nature of the addictive agent, such as psychoactive properties, pharmacokinetics and route of administration (Camí and Farré, 2003).

The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association [APA], 2013) describes the clues for the diagnosis of substance-related and addictive disorders. According to this last edition, substance-related disorders are divided into two groups: (1) substance use disorders, referring to changes produced in brain circuitry that can persist beyond detoxification, leading to cognitive, behavioural and psychological symptoms directly related to the substance use, and (2) substance-induced disorders, including intoxication, withdrawal and mental disorders induced by substances or medications. Therefore, the DSM-5 has combined the substance abuse and substance dependence categories, previously separated in the 4th edition, into a single substance use disorder measured on a continuum

from mild to severe, depending on the number of criteria endorsed, which are listed in Table 2.

Table 2. DSM-5 diagnostic criteria for substance use disorders.

A. Impaired control over substance use
1. The individual may take the substance in larger amounts or over a longer period of time than was originally intended.
2. The individual may express a persistent desire to cut down or regulate substance use and may report multiple unsuccessful efforts to decrease or discontinue drug use.
3. The individual may spend a great amount of time obtaining or using the substance or recovering from its effects.
4. Craving is manifested by the individual as an intense urge for the drug that may occur at any time but it is more likely to happen in an environment where the drug has been previously obtained or used.
B. Social, occupational, or recreational detriment
5. Recurrent substance use may result in a failure to fulfil major role obligations at work, school or home.
6. The individual may continue using the substance despite the appearance of social problems caused by the effects of the substance.
7. Important social, occupational, or recreational activities may be given up or reduced because of substance use.
C. Risky use of the substance
8. The individual uses the substance recurrently in physically hazardous situations.
9. Substance use is continued despite knowledge of having a psychological problem that is likely to have been caused by the substance.
D. Pharmacological criteria
10. A significant increase in the dose of the substance is needed to achieve the desired effect, or the effect produced with the usual dose is markedly reduced. This phenomenon is known as tolerance and it greatly varies between individuals and also between substances. It is important to consider that tolerance to different drug-induced effects could develop at different rates.
11. The individual reports physiological signs when blood or tissue concentrations of substance decline after a prolonged or heavy use of the substance. This is known as withdrawal syndrome. At this point the individual will likely consume the substance to relieve the symptoms. Withdrawal signs and symptoms vary greatly across the different classes of substance in part based on the effects of substance consumption.

2.1.2. Transition to drug addiction

The transition from controlled acute drug taking to drug addiction arises from drug-induced neuroadaptations in specific brain circuits that contribute to the long-lasting nature of the addictive disorder. During the initial development of addiction many relatively transient changes occur in the neuronal function that precede the emergence of a new behaviour due to the pharmacological effects of the drug itself. These neuroadaptations can persist for hours up to weeks of drug abstinence (Nestler, 2005). Nevertheless, repeated drug insults eventually induce relatively stable changes in synaptic physiology of brain circuits regulating cognitive and emotional responding to important environmental stimuli (Kalivas and O'Brien, 2008). These changes can remain virtually permanent, leading to the stable state of high vulnerability to relapse after long periods of abstinence that characterizes an addictive disorder.

2.1.2.1. Acute drug use: the reward stage

Drug consumption is often initiated as a result of social group pressure or curiosity. During this stage, acute drug use leads to a feeling of pleasure, usually referred as "high". These drug-induced rewarding effects are mainly achieved by the enhancement of the activity of the mesocorticolimbic circuit, which is composed of the VTA, containing the dopaminergic cell bodies, and the terminal areas in the NAc, amygdala and frontal and limbic cortices, where dopamine is released (Kelley, 2004; Wise, 2004) (Figure 5). Similar to motivationally relevant biological stimuli, all addictive drugs increase dopamine release within this circuit, albeit by different molecular mechanisms of action depending on their different molecular targets (Kelley, 2004; Koob, 1992; Nestler, 2005). Hence, the release of dopamine facilitates reward-induced learning, promoting recurrent drug-taking (Cardinal and Everitt, 2004; Wise, 2004). However, many authors agree that the precise role of dopamine in reward may be more complex than originally considered, and dopamine signalling might not translate into the subjective feeling of pleasure induced by a reward-related stimulus, but to the attraction and craving for it (Berridge, 2007, 2009; Evans *et al*, 2006).

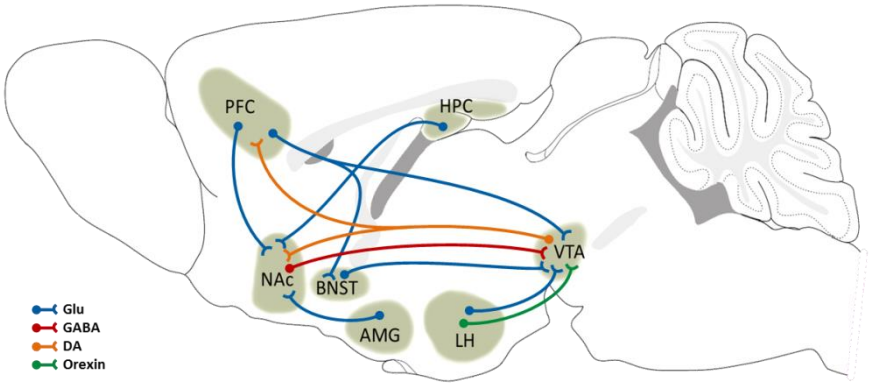


Figure 5. Mesocorticolimbic dopamine system. Simplified schema of the mesocorticolimbic system circuitry in rodent brain highlighting the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA). AMG, amygdala; BNST, bed nucleus of the stria terminalis; HPC, hippocampus; LH, lateral hypothalamus; PFC, prefrontal cortex (Adapted from Kauer and Malenka, 2007).

Diverse brain areas that are interconnected with the mesocorticolimbic dopaminergic system play also essential roles in acute drug reinforcement. These regions include the amygdala and related structures of the so-called "extended-amygdala" (comprising the central nucleus of the amygdala, the BNST and a transition zone in the shell subregion of the NAc), the hippocampus and the hypothalamus, among others (Alheid and Heimer, 1988; Nestler, 2005). Moreover, dopamine transmission is not the only mediator in the rewarding effects of all addictive drugs. Thus, an expanded and more complex network of neurochemical circuits around the dopamine mesolimbic system is thought to play also key roles in drug reward, including the opioid, endocannabinoid, glutamatergic and γ-aminobutiric acid (GABA)ergic systems (Koob and Volkow, 2010).

2.1.2.2. Repeated drug-taking: tolerance, sensitization and withdrawal stage

Every single drug exposure drives the mesocorticolimbic function beyond its physiological limits, overcoming normal homeostatic mechanisms for controlling dopamine release. Consequently, chronic drug exposure leads to neuroadaptations in the mesocorticolimbic system in an attempt to restore brain reward normal functioning (Nestler, 2005). Hence, baseline levels of dopamine function are progressively reduced, causing rewarding stimuli to be less effective at eliciting typical increases in dopaminergic

transmission. This phenomenon is named tolerance, and it might be developed towards biological stimuli and to the drug itself. Thus, individuals that have developed tolerance to the drug will need to increase the drug dose to re-experience its effects at the initial intensity (Nestler, 2005). At the same time, chronic drug exposure sometimes leads to sensitization of the dopamine system, a phenomenon characterized by greater increases in dopaminergic transmission in response to the drug itself or to drug-associated cues (Everitt and Wolf, 2002; Robinson and Berridge, 2000; Vezina and Leyton, 2009). This sensitization of dopamine system confers excessive incentive salience to the act of drug taking and to stimuli associated with drug taking, transforming ordinary 'wanting' into excessive drug craving and relapse (Robinson and Berridge, 2000). Despite being opposing processes, tolerance and sensitization can concurrently exist and likely involve different properties of the same dopaminergic neurocircuits.

Chronic drug-taking is characterized by the appearance of an aversive state when drug intake is discontinued, termed withdrawal syndrome, that results in recurrent drug use in order to avoid the negative consequences of drug abstinence (Koob and Le Moal, 1997, 2008b). All drugs of abuse can produce a motivational withdrawal syndrome characterized by dysphoria, irritability, and emotional distress. However, abrupt interruption of some particular drugs such as opiates, alcohol or sedative hypnotics use can also trigger an intense physical withdrawal (Koob and Le Moal, 1997, 2008b). Although the mechanisms underlying acute physical withdrawal seem to be drug-specific, acute withdrawal from all major drugs of abuse is characterized by a significant decrease in mesocorticolimbic dopaminergic activity (George *et al*, 2012), and the consequent reduced dopamine levels in the NAc contributes to the anhedonia associated with abstinence states. Another relevant mechanism involved in this stage is the activation of the brain stress systems. Thus, pharmacological blockade of CRF typically reverses the anxiogenic-like effects observed during acute withdrawal from all major drugs of abuse (Funk *et al*, 2006; George *et al*, 2007; Specio *et al*, 2008). In summary, although drug consumption is initially triggered by the positive reinforcing effects of addictive drugs, negative reinforcement is

responsible for maintaining drug use in order to avoid the negative affective state during abstinence (Koob, 2004).

2.1.2.3. Long-term abstinence: craving and relapse stage

The act of engaging drug-seeking after a period of drug abstinence is termed relapse, and a particularly troublesome aspect of drug addiction is that the vulnerability to relapse persists for years even in the absence of repeated drug use. The main stimuli recognized to trigger relapse in humans are: (1) the re-exposure to the drug originating the addiction or other drugs of abuse (De Wit, 1996), (2) the presence of drug-associated environmental cues (Carter and Tiffany, 1999), and (3) stressful situations or negative emotional states (Shiffman *et al*, 1996). Although different relapse-triggering stimuli involve different brain areas, drug-seeking reinstatement experiments in animals combined with functional imaging studies in addicts reveal that all of them share a common neurobiological scenario. Indeed, long-term drug-induced neuroadaptations in the corticostriatal glutamatergic transmission produce a disruption of information processing, decreasing the ability of addicts to regulate drug-seeking behaviours and increasing their vulnerability to relapse (Kalivas, 2009). Glutamatergic inputs from cortex and allocortex (e.g., amygdala and hippocampus) into the striatal motor circuit (including the dorsal striatum and the NAc) are critical for the execution of learned behaviours. As a behaviour is repeatedly executed, the role of glutamate projecting from the PFC and amygdala into the NAc becomes less important in favour of glutamate projecting from sensory motor cortical areas to the dorsal striatum (Everitt and Robbins, 2005). This transition from prefrontal circuitry to habit motor circuitry reduces the capacity of prefrontal executive function to intrude and disrupt the drug-seeking habit, translating into loss of control and compulsive relapse (Kalivas and O'Brien, 2008; Kalivas and Volkow, 2005).

2.2. Role of the orexin system in drug reward and addiction

Since the first functional studies relating orexin transmission to drug addiction published in 2005 (Boutrel *et al*, 2005; Harris *et al*, 2005), considerable knowledge has accumulated supporting a role for this system in the addictive properties of diverse drugs of abuse. The main

findings regarding orexin modulation of the rewarding effects and addictive properties of cocaine, opiates, alcohol and nicotine are detailed below.

2.2.1. Role of orexins in cocaine addiction

The contribution of the orexin system to the addictive properties of cocaine appears to be complex. Some evidence suggest that orexin transmission modulates, through OX1R signalling, the effects of cocaine in mesolimbic dopaminergic transmission. Thus, intra-VTA infusion of orexin-A enhanced cocaine-induced increased dopamine levels in the NAc, whereas OX1R antagonism, but not OX2R antagonism, caused the opposite effect (España *et al*, 2010, 2011; Prince *et al*, 2015). However, the primary rewarding properties of cocaine appear to be independent from orexin transmission. Thus, OX1R antagonism did not block the expression of a place preference conditioned by cocaine (Sharf *et al*, 2010a), and had no effect in cocaine self-administration (España *et al*, 2010, 2011; Smith *et al*, 2009; Zhou *et al*, 2012). In contrast, orexins contribute to cocaine-induced reinforcing effects when access to cocaine is restricted or under conditions that require higher effort to obtain the drug. Hence, the OX1R antagonist SB-334867 reduced cocaine self-administration in a discrete-trial procedure, where the number of drug infusions per hour is limited (España *et al*, 2010), and under a progressive-ratio schedule of reinforcement, which measures the motivation to obtain a drug infusion (Borgland *et al*, 2009; España *et al*, 2010). Consistently, central and intra-VTA infusion of orexin-A increased responding for cocaine in both experimental procedures (España *et al*, 2011), suggesting that the enhancement of orexin transmission in the VTA increases the reinforcing efficacy and the motivational properties of cocaine.

Orexins display also a crucial role in the reinstatement of a previously extinguished cocaine-seeking behaviour. Thus, central orexin-A infusion reinstates a previously extinguished cocaine-seeking behaviour (Boutrel *et al*, 2005; Wang *et al*, 2009). Consistently, systemic injection of the OX1R antagonist SB-334867 prevents cue- (Bentzley and Aston-Jones, 2015; Smith *et al*, 2009; Zhou *et al*, 2012), context- (Smith *et al*, 2010) and stress-induced reinstatement of cocaine-seeking (Boutrel *et al*, 2005). On

the contrary, cocaine-seeking elicited by cocaine-conditioned cues is independent of OX2R signalling (Smith *et al*, 2009).

2.2.2. Role of orexins in opioid addiction

The contribution of the orexin system to opioid-induced rewarding effects has been extensively reported. LH orexin neurons projecting to the VTA are activated in rats that exhibit consistent place preference conditioning by morphine, as observed by c-Fos studies (Harris *et al*, 2005; Richardson and Aston-Jones, 2012). Accordingly, morphine-elicited place preference and extracellular dopamine release in the NAc were abolished in mice that lacked the prepro-orexin gene (Narita *et al*, 2006). OX1R signalling throughout the mesolimbic system seems to be responsible of these findings, since either systemic, intra-VTA or intra-NAc blockade of this receptor attenuates the expression of morphine-induced place preference (Harris *et al*, 2005; Narita *et al*, 2006; Sadeghzadeh *et al*, 2015; Sharf *et al*, 2010a). In addition, OX1R antagonism decreases heroin intake in an operant self-administration paradigm in rats (Smith and Aston-Jones, 2012), and OX2R antagonism has similar effects when animals are subjected to a long-access heroin self-administration regime (Schmeichel *et al*, 2015), suggesting a role for both orexin receptors in opioid reinforcement.

The orexin system is also involved in somatic and negative affective symptoms of morphine withdrawal. Thus, orexin cells are activated in response to naltrexone- or naloxone-precipitated morphine withdrawal (Georgescu *et al*, 2003; Laorden *et al*, 2012; Sharf *et al*, 2008) and both naloxone-precipitated and spontaneous withdrawal increase orexin mRNA levels in the LH (Laorden *et al*, 2012; Zhou *et al*, 2006). Consistently, physical signs induced by naloxone-precipitated morphine withdrawal are attenuated in prepro-orexin knockout mice (Georgescu *et al*, 2003). OX1R signalling, particularly within the LC, appears to be necessary for the somatic expression of morphine withdrawal (Azizi *et al*, 2010; Laorden *et al*, 2012; Sharf *et al*, 2008), whereas OX2R within the paraventricular nucleus of the thalamus (PVT) seems to contribute to the negative affective component of morphine withdrawal (Li *et al*, 2011).

Few studies have evaluated the possible contribution of the orexin system in opioid relapse. Orexin-A injection into the VTA reinstated an extinguished morphine place preference in an OX1R-dependent manner (Harris *et al*, 2005). Accordingly, OX1R antagonism attenuated cue-induced, but not priming-induced reinstatement of heroin-seeking in an operant self-administration paradigm (Smith and Aston-Jones, 2012). Another study reported also a role for OX2R in opioid relapse, showing that blockade of OX1R or OX2R in the NAc attenuated stress-induced reinstatement of conditioned place preference for morphine, but neither of the orexin antagonists had any effect on morphine priming-induced reinstatement (Qi *et al*, 2013).

2.2.3. Role of orexins in alcohol addiction

A considerable number of studies support the contribution of orexins to the rewarding effects of alcohol, but the specific role of each orexin receptor subtype remains controversial. Rats treated with the OX1R antagonist SB-334867 displayed attenuated operant alcohol intake (Lawrence *et al*, 2006; Richards *et al*, 2008) and reduced ethanol preference on a two-bottle free-choice paradigm (Moorman and Aston-Jones, 2009). Moreover, SB-334867 administration reduced the breaking point in a progressive-ratio schedule of reinforcement, suggesting a role for OX1R in the motivation for alcohol consumption (Jupp *et al*, 2011a). In contrast, SB-334867 had no effect on acquisition and expression of ethanol-induced place preference in mice (Voorhees and Cunningham, 2011), and SB-408124, another OX1R antagonist, was ineffective in reducing ethanol self-administration in rats (Shoblock *et al*, 2011). These divergent results may be explained because different experimental procedures may examine different components of ethanol-induced reward, and hence, distinct neurobiological mechanisms. Recent reports support a role for OX2R in alcohol-induced reward. Thus, rats treated with diverse OX2R antagonists displayed reduced ethanol self-administration (Brown *et al*, 2013; Shoblock *et al*, 2011), decreased acquisition and expression of ethanol-induced place preference (Shoblock *et al*, 2011), and attenuated motivation for ethanol intake (Anderson *et al*, 2014). Moreover, local antagonism of OX2R, but not OX1R, in the PVT reduced ethanol intake in an intermittent access procedure (Barson *et al*, 2015).

Contribution of the orexin system to alcohol relapse appears to be more defined. Context- and cue-induced alcohol seeking activate LH orexin-expressing neurons (Dayas *et al*, 2008; Hamlin *et al*, 2007; Moorman *et al*, 2016). In agreement, OX1R antagonism, but not OX2R blockade, prevented cue-induced reinstatement of alcohol-seeking (Brown *et al*, 2013; Dayas *et al*, 2008; Jupp *et al*, 2011b; Lawrence *et al*, 2006; Martin-Fardon and Weiss, 2014). Local infusion of the OX1R antagonist SB-334867 in the VTA and the PFC also attenuated cue-induced reinstatement of ethanol-seeking, suggesting that OX1R located in these brain regions are part of the circuit driving cue-mediated ethanol-seeking behaviour (Brown *et al*, 2015). Consistent with preclinical data, recent studies in alcohol-dependent patients report certain correlation between orexin plasma concentration levels and withdrawal and craving severity, suggesting a role of orexins in the affective dysregulation that appears during these stages (von der Goltz *et al*, 2011; Ziółkowski *et al*, 2015).

2.2.4. Role of orexins in nicotine addiction

Growing evidence suggests that orexin transmission may influence the addictive properties of nicotine (Kenny, 2011). Orexin neurons of the LH show increased Fos expression upon acute nicotine injections (Pasumarthi *et al*, 2006), and chronic nicotine administration enhanced orexin peptide and receptor mRNA levels in the rat hypothalamus (Kane *et al*, 2000). OX1R signalling contributes to nicotine reinforcement and motivation to seek the drug, since OX1R antagonism decreased intravenous nicotine self-administration in rats and lowered the number of nicotine infusions earned under a progressive ratio schedule (Hollander *et al*, 2008; LeSage *et al*, 2010). Conversely, pre-treatment with an OX2R antagonist did not modify these behavioural responses, suggesting that nicotine reinforcement and motivation are OX2R-independent (Uslaner *et al*, 2014). Evidence from human studies also points to a role of orexin transmission in tobacco addiction. Thus, stroke-associated damage to the insular cortex in human smokers resulted in spontaneous cessation of the smoking habit (Naqvi *et al*, 2007). Consistent with this, intra-insular infusion of the OX1R antagonist SB-334867 into the insular cortex decreased nicotine intake in rats (Hollander *et al*, 2008), further

supporting the modulation of nicotine rewarding effects through orexin signalling in the insular cortex.

The orexin system also seems to participate in nicotine-seeking behaviours. Indeed, central infusion of orexin-A reinstates a previously extinguished nicotine-seeking behaviour in mice in an OX1R-dependent and CRF-independent manner (Plaza-Zabala *et al*, 2010). In addition, administration of SB-334867, but not the OX2R antagonist TCS-OX2-29, attenuated cue-induced reinstatement of nicotine-seeking through PKC signalling pathway (Plaza-Zabala *et al*, 2013). However, another OX2R antagonist, 2-SORA 18, has been shown to effectively block nicotine-seeking triggered by nicotine-associated cues (Uslaner *et al*, 2014). Additional research will be needed to disentangle the role of orexin receptor signalling in relapse of nicotine seeking.

On the other hand, a role for the orexin system in the expression of nicotine withdrawal has been reported. Thus, systemic administration of the OX1R antagonist SB-334867, but not the OX2R antagonist TCS-OX2-29, attenuated the somatic signs of mecamylamine-precipitated nicotine withdrawal in mice (Plaza-Zabala *et al*, 2012a). In addition, the increase in Fos expression that occurred in the PVN upon nicotine withdrawal precipitation was dependent on OX1R, and local infusion of SB-334867 into this brain area attenuated the somatic manifestations of withdrawal (Plaza-Zabala *et al*, 2012a).

In summary, strong evidence supports that orexin transmission does not only contributes to the primary reinforcing and motivational properties of drugs of abuse, but also to development of aversive states during drug withdrawal and to processes that drive relapse to drug seeking. As mentioned, several studies have examined the participation of orexins in addiction to cocaine, opioids, alcohol and nicotine, but the possible role of the orexin system in the addictive properties of cannabis, one of the most commonly used illicit drugs, has not been evaluated so far.

2.3. Cannabis addiction

2.3.1. Cannabis use: a double-edged sword

Cannabis is a generic term used for preparations derived from any of the genus cannabis plants, most commonly *Cannabis sativa*. Cannabis or hemp plant is among the earliest plants cultivated by man. Although it was initially used in the ancient China as a source of textile fibre (Li, 1973), later civilizations from the regions of India and Arabia widely took advantage of other varieties of *Cannabis sativa* for its curative and psychoactive properties (Kalant, 2001). Early documents from these regions report multiple medicinal uses of cannabis, such as analgesic, anxiolytic and anticonvulsant, but also with recreational purposes due to its pleasurable and relaxing effects (Zuardi, 2006). However, the manifestation of hallucinations, dysphoria and even addictive-like states upon cannabis consumption are also mentioned in diverse antique texts, denoting that awareness of its negative consequences is equally old (Zuardi, 2006). At the present time, the use of cannabis for medical purposes has become of increasing interest (Grotenhermen and Müller-Vahl, 2012). A formal evidence base for several medical indications is gradually building, and subsequently several countries have started legalising possession of small amounts of marijuana for medical purposes (Marcoux *et al*, 2013). At the same time, marijuana and other illicit cannabis preparations are widely used recreationally and represent a major public health concern. Although therapeutic potential for medical cannabis cannot be ignored, it represents a double-edged sword since cannabis comes to be increasingly perceived as safe by the public (Cook *et al*, 2015).

2.3.1.1. Cannabis use, abuse and dependence

Cannabis preparations, such as marijuana and hashish, are the most consumed illicit drugs worldwide. According to the latest European Drug Report (European Monitoring Center for Drugs and Drug Addiction [EMCDDA], 2015), it is estimated that 78.9 million European adults (15 to 64 years old) have consumed cannabis during their lifetime, which represents almost a quarter of this European population. It is estimated that almost 1 % of European adults are daily or almost daily cannabis

users, and around three-quarters of these are aged between 15 and 34 years. Currently, cannabis is the drug most frequently reported as the principal reason for entering drug treatment for the first time in Spain, and cannabis-related hospital emergencies have risen during the last years, reaching a 33% of all drug-related emergencies. Cannabis consumption has been associated with low academic achievement, unemployment, violence and risk for developing psychiatric disorders, especially in early onset cases (Ferdinand *et al*, 2005; Friedman *et al*, 2001; Hall, 2006).

The generally preferred route of cannabis administration is inhalation of smoke, in co-use with tobacco. Acute effects last for approximately two to three hours and are often described as a pleasant and relaxing experience, characterized by euphoria, sedation, and increased perception of external stimuli. Users typically experience increased appetite, tachycardia and bronchodilation (Karila *et al*, 2014). Additionally, cannabis use produces diverse acute adverse effects, including dysphoria, anxiety, panic reactions, and sometimes positive psychotic symptoms (e.g., hallucinations, delusions of persecution) (Hall, 2015; Johns, 2001). Acute cannabis also impairs short-term memory and attention, motor coordination, and reaction time (Hall, 2015; Karila *et al*, 2014), doubling the risk of a motor vehicle accident (Hartman and Huestis, 2013). Chronic cannabis use produces neurocognitive deficits that persist for several days or weeks after cessation of cannabis intake, and memory and attention impairments may persist and worsen with increasing years of use and with the initiation of use during adolescence (Volkow *et al*, 2014). High-dose, daily use can also give rise to a chronic intoxication syndrome, characterized by apathy, confusion, depression and paranoia (Kalant, 2004). Other long-term consequences of regular cannabis use include impaired respiratory function, cardiovascular disease, and naturally, cannabis abuse and dependence (Hall and Degenhardt, 2014; Volkow *et al*, 2014). The lifetime risk of dependence among all cannabis users is estimated at about 9% and increases to 17% among those who initiate use in adolescence (Hall and Degenhardt, 2014; Le Strat *et al*, 2015).

From a clinical perspective, DSM-5 now recognizes a cannabis-related disorders category that includes, among others, cannabis use disorder, cannabis intoxication and cannabis withdrawal. Similar to other drug

addictions, cannabis use disorder is a chronic condition characterized by repeated attempts to quit followed by relapse. Repeated exposure to cannabinoids results in tolerance to the subjective and performance-impairing effects of cannabis, and cessation of chronic drug use may induce the appearance of a withdrawal syndrome mainly characterized by negative affective states (Panlilio *et al*, 2015). Over time, cannabis withdrawal has gained recognition as a clinically significant phenomenon, being subsequently added to DSM-5 and included as one of the criterions for cannabis use disorder (Haney, 2005; Hasin *et al*, 2013). The DSM-5 diagnostic criteria for cannabis withdrawal are listed in Table 3.

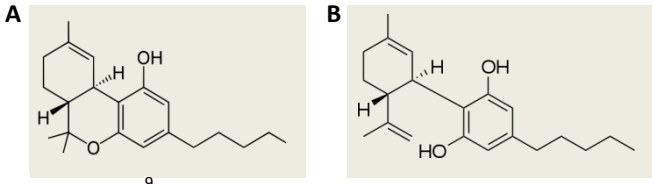
Table 3. DSM-5 diagnostic criteria for cannabis withdrawal.

A. Cessation of cannabis use that has been heavy and prolonged (i.e., usually daily or almost daily use over a period of at least a few months).
B. Three (or more) of the following signs and symptoms develop within approximately 1 week after criterion A: 1. Irritability, anger, or aggression 2. Nervousness or anxiety 3. Sleep difficulty (e.g., insomnia, disturbing dreams) 4. Decreased appetite or weight loss 5. Restlessness 6. Depressed mood 7. At least one of the following physical symptoms causing significant discomfort: abdominal pain, shakiness/tremors, sweating, fever, chills, or headache.
C. The signs or symptoms in criterion B cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
D. The signs or symptoms are not attributable to another medical condition and are not better explained by another mental disorder, including intoxication or withdrawal from another substance.

2.3.2. Exogenous cannabinoids

The main active compounds isolated from cannabis, called phytocannabinoids, are Δ^9-tetrahydrocannabinol (THC), cannabidiol, Δ^8-tetrahydrocannabinol and cannabinol (Pertwee, 2005). More than other 80 cannabinoids with closely related structures and physical properties

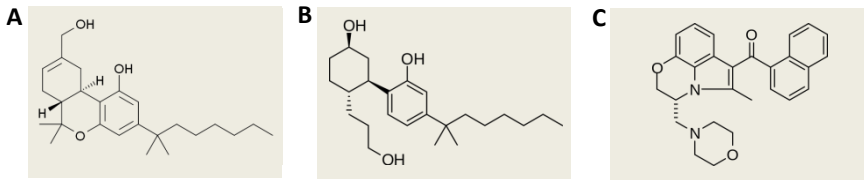
have also been identified in the hemp plant, such as cannabichromene, cannabigerol, cannabicyclol and cannabitriol (Elsohly and Slade, 2005). Among them, THC is the main psychoactive component in the cannabis extracts (Gaoni and Mechoulam, 1964) (Figure 6A), and is considered to be a partial agonist due to its moderate cannabinoid receptor affinity and potency (Pertwee *et al*, 2010). Another abundant phytocannabinoid, cannabidiol (Figure 6B), lacks the psychoactive effects and produces anti-inflammatory responses (Iuvone *et al*, 2009).



Δ^9-THC CBD

Figure 6. Chemical structure of the most relevant phytocannabinoids. Δ^9-THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol.

Beside these natural compounds, a series of synthetic cannabinoids have been designed displaying different selectivity profiles for the cannabinoid receptors. According to their chemical structure, synthetic agonists are classified as classical, non-classical, and aminoalkylindoles (Pertwee *et al*, 2010). The classical group includes all dibenzopyran derivatives of THC, such as HU-210 (Figure 7A) and nabilone, both of which show higher cannabinoid receptor affinity, intrinsic activity and potency than THC. The non-classical group consists of bicyclic and tricyclic analogues of THC that lack the pyran ring, among which the best known member is CP-55,940 (Figure 7B). The compounds of the aminoalkylindole group present a structure completely different to THC, and their most representative member is WIN55,212-2 (Figure 7C) (Pertwee *et al*, 2010).



HU-210 CP-55,940 WIN55,212-2

Figure 7. Chemical structure of the most representative members of each synthetic cannabinoid agonist group.

Selective antagonists for the different cannabinoid receptors have been synthesized, since the manipulation of cannabinoid signalling could be advantageous due to the wide range of effects regulated by the endocannabinoid system. Several compounds such as SR141716A (rimonabant), AM251, and MK0364 (taranabant) can block agonist-induced activation of cannabinoid type 1 (CB1R) receptor in a competitive manner (Gatley *et al*, 1996; Lin *et al*, 2006; Rinaldi-Carmona *et al*, 1994). In some cases, these compounds have been found to act as inverse agonists as they induce opposite effects to those produced by CB1R agonists (Fong *et al*, 2007). This fact has prompted the development of neutral CB1R antagonists, such as NESS-0327, which only produce the blockade of agonist-induced effects without any other intrinsic effect (Ruiu *et al*, 2003). On the other hand, some compounds can selectively block cannabinoid type 2 (CB2R) receptor activation, including AM630 and SR144528 (Pertwee *et al*, 1995; Rinaldi-Carmona *et al*, 1998). Both are CB2R-selective competitive antagonists and can behave as inverse agonists. Neutral antagonists that selectively target CB2R have not been developed yet.

2.3.2.1. The K2/Spice phenomenon

Synthetic cannabinoids were originally developed for their legitimate use as tools in scientific and medical research. Unfortunately, clandestine laboratories began illegally synthesizing some of the compounds for illicit use and sale (Wiley *et al*, 2011). These products, deceptively marketed as natural herbal incense highs, rapidly emerged as popular drugs of abuse in 2004 when commercial preparations branded as “K2” in the United States or as “Spice” in Europe became readily available online and in “headshops” (Seely *et al*, 2011). It was not until 2008 that synthetic cannabinoids were formally detected in individuals by European institutions (EMCDDA, 2009). Since then, reports of Spice abuse have increased worldwide exponentially (Brents and Prather, 2014).

Spice preparations consist on dried plant material sprayed with a mixture of synthetic cannabinoids, and is typically consumed by smoke inhalation (Musah *et al*, 2012). JWH-018, CP-47,497 and HU-210, as well as other analogues, represent examples of originally research compounds identified in early Spice samples (Wiley *et al*, 2011). However, there are

more than a hundred of these psychoactive compounds on the market because clandestine chemists steadily produce novel cannabimimetic designer drugs to replace synthetic cannabinoids as they are banned (Ernst *et al*, 2012; EMCDDA, 2015; Rosenbaum *et al*, 2012). One challenge in the control of these psychotropic products is the identification of novel synthetic cannabinoids in Spice products, for which there are no genuine standards (Brents and Prather, 2014). The highly variable and unpredictable composition of Spice products, as well as their higher potency and affinity for endogenous targets than cannabis compounds, entail potentially harmful consequences that might not be perceived by Spice users (Castaneto *et al*, 2014). Spice preparations produce physiological and psychoactive effects similar to THC, but with greater intensity, resulting in medical and psychiatric emergencies. Human adverse effects include nausea and vomiting, tachycardia, anxiety, hallucinations, and cognitive impairment, among others. Long-term or residual effects are unknown, but their abuse liability is undeniable, and some chronic users have experienced withdrawal symptoms when they stopped drug intake (Zimmermann *et al*, 2009).

Although the potential addiction to synthetic cannabinoids is beyond the primary objectives of this thesis, the neurobiological substrates underlying the effects of Spice products may be similar to those involved in cannabis effects. Since we employed a synthetic cannabinoid to model cannabinoid reward, our findings might hopefully contribute to Spice-related research as well.

2.3.3. The endocannabinoid system

Albeit the extensive consumption of cannabis derivatives over thousands of years, it was not until the 1980s when it was unravelled that cannabinoid compounds exert their biological effects through the activation of specific endogenous receptors (ever since termed cannabinoid receptors), instead of by altering the cellular membrane permeability as it was formerly believed (Devane *et al*, 1988; Howlett, 1984, 1985; Munro *et al*, 1993). This discovery was followed by the identification of the endogenous ligands of cannabinoid receptors, which were referred to as endocannabinoids (Devane *et al*, 1992; Mechoulam *et al*, 1995). Hence, the endocannabinoid system consists of

endocannabinoids, their GPCR-family receptors and the enzymatic machinery that synthesizes and degrades endocannabinoids. Therefore, exogenous cannabinoids act through hijacking of the endocannabinoid system, which represents the major site of action of THC and other cannabinoids, the biological effects induced by these compounds is directly linked with the neuroanatomical distribution and physiological role of this endogenous system. The endocannabinoid system acts as a retrograde modulator of several brain neurotransmitters and is widely present throughout the entire brain. This system is involved in a wide variety of biological functions, including brain development, control of energy expenditure, motivation, pain perception, and stress coping, among others (Chen, 2015).

2.3.3.1. Cannabinoid receptors

Cannabinoids exert their pharmacological actions through the activation of at least two distinct cannabinoid receptors: CB1R and CB2R. CB1R was the first cloned and characterized cannabinoid receptor (Matsuda *et al*, 1990), abundantly expressed throughout the CNS (Herkenham *et al*, 1991). CB2R was identified three years later and was considered a peripheral receptor, since it was initially found in the spleen (Munro *et al*, 1993). Both belong to the GPCR family and are mainly coupled to Gi/o protein. Diverse studies also point to the existence of other receptors that bind cannabinoid ligands, such as G protein-coupled receptor 55 (GPR55) (Pertwee, 2007), the sphingosine-1-phosphate lipid receptors GPR3, GPR6 and GPR12 (Kostenis, 2004; Yin *et al*, 2009), the peroxisome proliferator-activated receptor (PPAR) (O'Sullivan, 2007), or the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Di Marzo and De Petrocellis, 2010).

Cannabinoid receptor type 1

CB1R is one of the most abundant seven-transmembrane domain receptors in the CNS and constitute the major cannabinoid receptor involved in the psychoactive effects of THC and other cannabinoid ligands. Its distribution has been well characterized both in rodents (Figure 8) (Herkenham *et al*, 1991; Tsou *et al*, 1998) and in humans (Burns *et al*, 2007; Terry *et al*, 2010; Westlake *et al*, 1994). The highest density of CB1R

has been observed in the basal ganglia, cerebellum, and hippocampus. These receptors have also been found in cortex, amygdala, thalamus and hypothalamus, among other brain regions (Herkenham *et al*, 1991). CB1R is also expressed in peripheral tissues, including the retina, gonads, peripheral neurons, adipocytes, heart, lung, liver, adrenal gland, and immune and vascular system (Pertwee *et al*, 2010). At the cellular level, CB1R expression is mainly restricted to presynaptic terminals, where they modulate the release of multiple excitatory and inhibitory neurotransmitters, usually by promoting the inhibition of their release (Wilson and Nicoll, 2002).

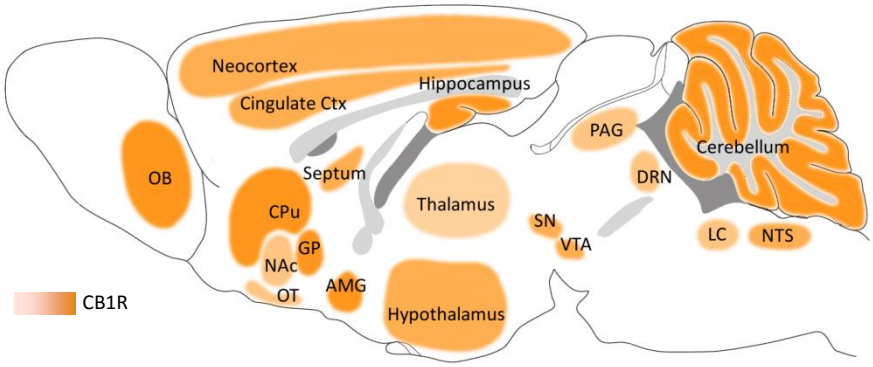


Figure 8. Distribution of CB1R. Sagittal section of mouse brain showing schematic CB1R location (different shading density indicates expression level). AMG, amygdala; ctx, cortex; Cpu, caudate-putamen; DRN, dorsal raphe; GP, globus pallidus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; SN, substantia nigra; VTA, ventral tegmental area. (Adapted from Herkenham *et al*, 1991, and Tsou *et al*, 1998).

Cannabinoid receptor type 2

CB2R are primarily located in the immune system including the spleen, thymus and immune cells, and are deeply involved in inflammatory processes (Walter and Stella, 2004). Early studies showed that CB2R was absent in CNS neurons, and that healthy brain tissue does not express CB2R (Munro *et al*, 1993). However, further research suggests that CB2R exists in neurons on the brainstem, dorsal root ganglia, lumbar spinal cord, and possibly on the cerebellum (Gong *et al*, 2006; Onaivi *et al*, 2006; Van Sickle *et al*, 2005). In addition, CB2R may be upregulated under neuroinflammatory conditions in certain cell populations within the brain, such as microglial cells (Carlisle *et al*, 2002; Stella, 2010; Walter *et al*,

2003). Interestingly, recent studies suggest that beside their role in neuroinflammation, CB2R also controls the rewarding properties of diverse addictive drugs, such as cocaine, alcohol and nicotine (Aracil-Fernández *et al*, 2012; Navarrete *et al*, 2013; Ortega-Álvaro *et al*, 2015; Xi *et al*, 2011).

2.3.3.2. Endocannabinoids

The discovery of the cannabinoid receptors impelled the research to identify endogenous cannabinoid receptor ligands, collectively known as endocannabinoids. The first ligand identified, N-arachidonoylethanol-amide, was named anandamide based on the Sanskrit word *ananda* that means "bliss" (Figure 9A) (Devane *et al*, 1992). Anandamide behaves as a partial agonist at both CB1R and CB2R, although presents lower affinity for CB2R, and binds also to TRPV1 receptor (Cristino *et al*, 2008). Shortly thereafter, the second major endocannabinoid was also identified: 2-AG (Figure 9B) (Mechoulam *et al*, 1995; Sugiura *et al*, 1995). 2-AG concentration in the brain is much higher than anandamide, and acts as full agonist for both CB1R and CB2R with higher potency than anandamide (Reggio, 2010). Beside these molecules, other putative endocannabinoids have also been identified, such as 2-arachidonylglycerolether (Hanus *et al*, 2001) and O-arachidonoylethanolamine (Porter *et al*, 2002). Despite the ability of these endogenous lipids to bind to cannabinoid receptors, their functional relevance remains to be elucidated.

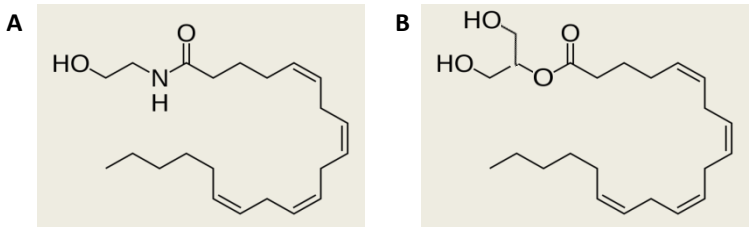


Figure 9. Chemical structure of the most well-known endocannabinoids. 2-AG, 2-arachidonoylglycerol.

Unlike the majority of neurotransmitters, anandamide and 2-AG are not stored in presynaptic vesicles, but rather synthesized and released on demand in the postsynaptic terminals in an activity-dependent manner (Di

Marzo *et al*, 2005). Once released from the postsynaptic neurons, endocannabinoids travel backward across synapses and activate CB1R on presynaptic terminals, acting as rapid retrograde synaptic messengers in order to produce a transient decrease of the release of other neurotransmitters (Figure 10) (Ohno-Shosaku *et al*, 2001; Wilson and Nicoll, 2002). According to this rapid neuromodulatory effect, endocannabinoid availability in the synaptic cleft needs to be finely regulated through balancing its biosynthesis and degradation.

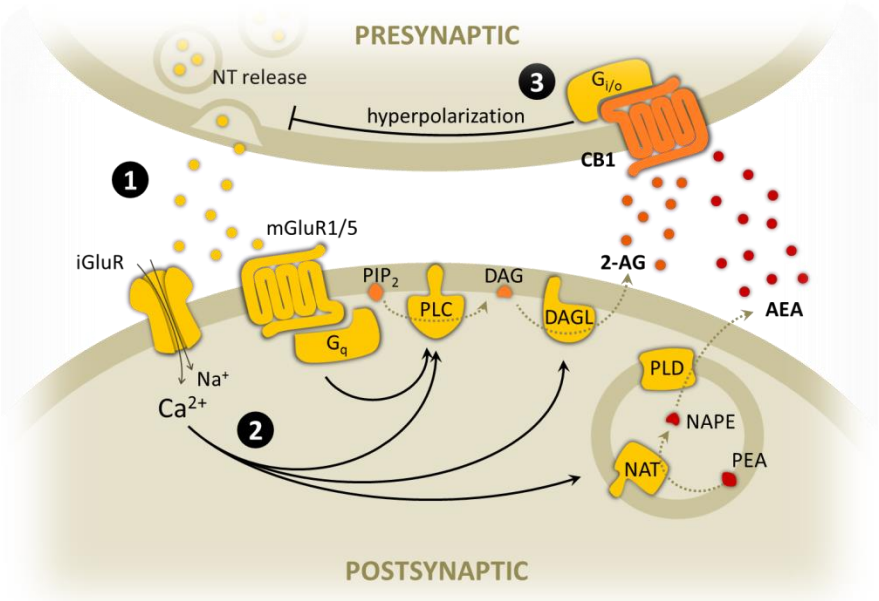


Figure 10. Endocannabinoid-mediated retrograde inhibition of neurotransmitter release in glutamatergic transmission. (1) Glutamate is released from presynaptic terminals and stimulates both ionotropic and metabotropic glutamate receptors, leading to postsynaptic depolarization through Ca^{2+} entrance and Gq-protein activation. (2) High Ca^{2+} concentration stimulates endocannabinoid biosynthesis. The enzymes for 2-AG biosynthesis –phospholipase C (PLC) and a specific diacylglycerol lipase (DAGL)– seem to be mostly localized on the plasma membrane on postsynaptic neurons, while anandamide biosynthetic enzymes –N-acyl transferase (NAT) and a selective phospholipase D (PLD)– are located on intracellular membranes. (3) Endocannabinoids are released to the synaptic cleft and act retrogradely on the CB1 located at the presynaptic terminals to produce transient decrease of neurotransmitter release. 2-AG; 2-arachidonoylglycerol; AEA, anandamide; DAG, diacylglycerol; NAPE, N-arachidonoyl-phosphatidyl-ethanolamine; PEA, phosphatidylethanolamine; PIP2, phosphatidylinositol bisphosphate. (Adapted from Di Marzo *et al*, 2004).

2.3.3.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids

All endogenous cannabinoids are lipid derivatives containing arachidonic acid, produced by the hydrolysis of phospholipid precursors (Figure 10). Anandamide is principally synthetized as a consequence of the hydrolysis of its phospholipid precursor N-arachidonoyl-phosphatidylethanolamine by the action of a specific phospholipase D (Di Marzo *et al*, 1994, 2005). 2-AG results from the hydrolysis of diacylglycerol by a DAGL (Di Marzo *et al*, 2005). Despite their lipophilic nature, endocannabinoids are removed from the synaptic cleft and taken up by the cell following their release and upon activation of their molecular targets. This process occurs via rapid diffusion through the cell membrane, although it might be facilitated by the presence of specific transporter proteins by a mechanism not completely characterized (Hillard and Jarrahian, 2003; Nicolussi and Gertsch, 2015). After its reuptake in the cell, endocannabinoids are degraded by the effect of specific hydrolases. Anandamide is hydrolysed to arachidonic acid and ethanolamine by fatty acid amine hydrolase (FAAH) (Cravatt *et al*, 1996), while 2-AG is mainly hydrolysed by the monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol (Dinh *et al*, 2002a, 2002b). Both are intracellular enzymes, but FAAH is primarily expressed in the soma and dendrites of postsynaptic neurons (Egertová *et al*, 2003), whereas MAGL is expressed in presynaptic terminals (Gulyas *et al*, 2004).

2.3.3.4. Cannabinoid cellular signalling

Stimulation of cannabinoid receptors produces a wide variety of effects through the activation of diverse signal transduction pathways (Figure 11) (Bosier *et al*, 2010). Both CB1R and CB2R exert their reported biological effects by activating heterotrimeric Gi/o type G proteins. Through coupling to Gαi/o, CB1R activation mediates the inhibition of AC, with subsequent reduction in cAMP levels and protein kinase A activity (Howlett, 2005). In addition, CB1R coupling to Gβγi/o can stimulate the phosphorylation and activation of various members of the MAPK family, including ERK1/2, p38 and c-Jun N-terminal kinase (Bouaboula *et al*, 1995; Howlett, 2005). CB1R stimulation can also activate other kinase signalling

cascades, such as the phosphoinositide 3-kinase pathway, glycogen synthase kinase 3 and PKC (Bouaboula *et al*, 1995; Gómez del Pulgar *et al*, 2000; Hillard and Auchampach, 1994; Ozaita *et al*, 2007), indicating the relevance of changes on protein phosphorylation in the mechanism of action of these compounds. CB1R also modulates the activity of several ion channels, including the activation of the inward-rectifying K^+ channels, and the inhibition of N-type and P/Q-type Ca^{2+} channels, triggering the repolarization of the plasmatic membrane and hindering neurotransmitter release (Bosier *et al*, 2010; Deadwyler *et al*, 1995; Howlett *et al*, 2002; Vásquez *et al*, 2003). Beside Gi/o protein association, CB1R may also couple to Gs and Gq under certain circumstances (Glass and Felder, 1997; Lauckner *et al*, 2005). Finally, it is worth mentioning that the lipid composition of the cellular membrane in the surroundings of the receptor, and particularly cholesterol content, seems to be critical for the regulation of signal transduction pathways triggered upon CB1R stimulation (Maccarrone, 2010).

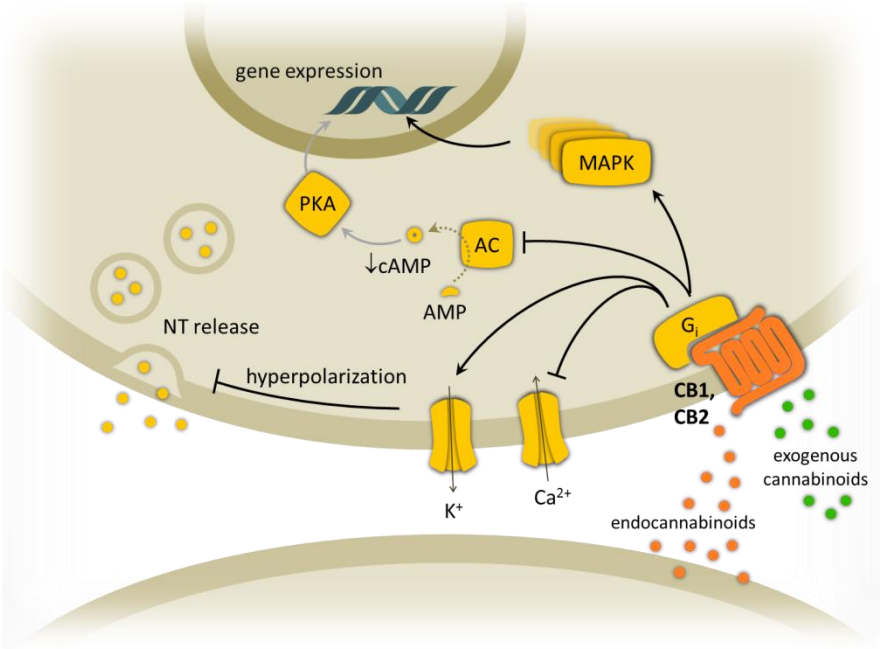


Figure 11. Main cannabinoid receptor signalling pathways. Both CB1R and CB2R are associated with Gi/o-dependent inhibition of AC activity and activation of the different MAPK cascades. CB1R negatively regulate voltage-gated Ca^{2+} channels and positively regulate inwardly rectifying K^+ channels, thereby inhibiting neurotransmitter release. AC, adenylyl cyclase; MAPK, diverse members of the mitogen-activated protein kinase cascade; PKA, protein kinase A. (Adapted from Bosier *et al*, 2010).

2.3.3.5. Physiological functions of the endocannabinoid system

Substantial research has consolidated our view on the endocannabinoid system as major contributor in the control of the synaptic homeostasis and in the proper development of brain functions. Thus, this neuromodulator system regulates synapse formation and remodelling (Harkany *et al*, 2008; Kano *et al*, 2009), and diverse processes involved in neuronal development, including neuronal survival, differentiation, proliferation and migration (Galve-Roperh *et al*, 2013; Harkany *et al*, 2008; Rueda *et al*, 2002). The extensive distribution of the endocannabinoid system in the CNS and numerous peripheral tissues correlates with its role as modulator of several physiological functions. The presence of CB1R in the basal ganglia and cerebellum has been related to fine control of motor coordination and cerebellar learning performance (Fernández-Ruiz and Gonzáles, 2005; Kishimoto and Kano, 2006). The endocannabinoid system also controls nociception under diverse sorts of acute and chronic pain (Maldonado *et al*, 2016; La Porta *et al*, 2014). Importantly, this neuromodulatory system ensures an appropriate reaction to stressful events, regulating anxiety and fear responses (Lutz *et al*, 2015). It has also been associated with the modulation of emotions and motivation (Mechoulam and Parker, 2013), reward processing and addiction (Maldonado *et al*, 2006; Parsons and Hurd, 2015). CB1R expression in the hippocampus has been widely investigated because of the effects of cannabis on learning and memory (Kano *et al*, 2009; Puighermanal *et al*, 2009). Acting at peripheral level, the endocannabinoid system modulates the immune and cardiovascular systems, controls gastrointestinal motility and metabolism, and regulates the function of the liver, the adipose tissue or the reproductive system, among others (Grotenhermen and Müller-Vahl, 2003; Watkins and Kim, 2014).

2.3.4. Behavioural effects of cannabinoids

The numerous physiological functions of the endocannabinoid system are directly linked with the wide variety of pharmacological effects produced by exogenous cannabinoids. This section focuses on the main behavioural alterations induced by cannabinoid compounds that have been evaluated in our studies, including the tetrad test (the specific trademark of

psychoactive cannabinoids), and the anxiety-like and amnesic-like effects. The rewarding effects of cannabinoids, also evaluated in our work, are addressed in detail in section *2.3.5. Behavioural models to evaluate cannabinoid-induced reward.*

The cannabinoid tetrad test

The cannabinoid tetrad is a battery of *in vivo* behavioural tests that are sensitive to THC and other psychoactive cannabinoids (Little *et al*, 1988). In rodents, four different effects are characteristically produced by prototypical cannabinoid agonists: hypolocomotion, hypothermia, antinociception and catalepsy. These effects are reversed by the selective CB1R antagonist rimonabant, providing evidence for the involvement of CB1R in these behaviours (Fox *et al*, 2001). The administration of exogenous anandamide or inhibitors of the endocannabinoid degradation has been shown to produce similar effects, although with much lower potency and duration (Crawley *et al*, 1993; Long *et al*, 2009; Smith *et al*, 1994).

Among the four effects of cannabinoid agonists included in the tetrad test, the antinociceptive properties, and in a lower extent the hypothermic properties, are the most relevant for their possible therapeutic application in humans. Probably due to the presence of cannabinoid receptors in multiple regions involved in nociceptive responses (Hohmann, 2002), cannabinoid compounds present antinociceptive effects that are exerted at supraspinal, spinal and peripheral levels (Guindon and Hohmann, 2009; Walker and Hohmann, 2005). Cannabinoids also produce antinociceptive effects in different types of pain (Guindon and Hohmann, 2009; Hohmann and Suplita, 2006), although these effects differ depending on the dose, the compound and the test used. Accordingly, blockade of CB1R can produce hyperalgesia under specific experimental conditions, supporting a physiological role of these receptors on nociception (Guindon and Hohmann, 2009). In addition, CB2R also regulates neuropathic (Racz *et al*, 2008), inflammatory (Pini *et al*, 2012), and osteoarthritic pain (La Porta *et al*, 2013). On the other hand, the hypothermic properties of cannabinoids have partially contributed to their increasing interest as possible neuroprotectors in medical conditions that present ischemic and other types of brain injury,

including cardiac arrest and neonatal hypoxia-isquemia (Hassell *et al*, 2015; Mulder and Geocadin, 2014). Indeed, the administration of different synthetic cannabinoids leaded to improved survival and neurological outcomes in a rat model of cardiac arrest (Ma *et al*, 2014; Weng *et al*, 2012). This effect was mediated by CB1R (Weng *et al*, 2012) and blocked by preventing hypothermia with an external heating source (Ma *et al*, 2014).

Anxiety-like effects

Considerable data exist on the direct effects of exogenous and endogenous cannabinoids on anxiety (Viveros *et al*, 2005). In humans, THC may cause either euphoria and relaxation or dysphoria and anxiety (D'Souza *et al*, 2004; Wade *et al*, 2003). These biphasic properties of cannabinoids have also been observed in experimental animals, and depend on the dosage, genetic background and environmental context (Moreira *et al*, 2009). Thus, THC elicits anxiolytic-like responses at low doses, whereas higher doses induce anxiogenic-like effects (Rubino *et al*, 2008a; Viveros *et al*, 2005). Interestingly, several studies in animals have revealed that increased levels of endogenous cannabinoids produce anxiolytic-like responses. Thus, systemic administration of selective FAAH or MAGL inhibitors *(Kathuria et al, 2003; Moreira et al, 2008; Sciolino et al*, 2011), as well as intra-PFC infusion of an anandamide derivative (Rubino *et al*, 2008b), had anxiolytic properties in different anxiety tests. It seems that anandamide might be acting through CB1R, while 2-AG may act through CB2R to elicit these anxiolytic-like responses (Busquets-Garcia *et al*, 2011). The anxiety-like phenotype shown by CB2R-overexpressing mice supports this role of CB2R on the modulation of anxiety behaviour (García-Gutiérrez and Manzanares, 2011).

Amnesic-like effects

Cannabis consumption impairs cognitive performance, attention, working memory and cognitive flexibility in humans (Lundqvist, 2005). Transient impairment in short-term episodic memory and deficient memory consolidation has also been reported under the effects of THC (Hall *et al*, 1999; Ilan *et al*, 2004; Lundqvist, 2005). Likewise, acute administration of THC and other CB1R agonists impairs short-term and working memory in

different animal models (Mechoulam and Parker, 2013; Varvel *et al*, 2001). In addition, acute THC produced CB1R-dependent amnesic-like effects in the novel object recognition test (Puighermanal *et al*, 2009), which were also sustained during chronic THC exposure (Puighermanal *et al*, 2013). Moreover, rats exposed to chronic THC during adolescence display impaired spatial working memory even 30 days after the treatment (Rubino *et al*, 2009). The detriment in memory produced by cannabinoids appears to be directly related with their action in the hippocampus. Thus, administration of CB1R agonists directly into the hippocampus produces impairments in working memory performance in a wide variety of tests (Abush and Akirav, 2010; Clarke *et al*, 2008; Suenaga *et al*, 2008; Wegener *et al*, 2008). It seems that the balance between GABAergic and glutamatergic transmission induced by CB1R stimulation might be crucially involved in these disrupting effects of THC, at least in recognition memory (Puighermanal *et al*, 2009).

2.3.5. Behavioural models to evaluate cannabinoid-induced reward

Compared to other addictive drugs, such as opioids, psychostimulants and alcohol, cannabis derivatives had been classically considered to have less potential to produce dependence in humans, probably because early studies reported that cannabis derivatives do not induce physical dependence or an abstinence syndrome (Hollister, 1986). However, overwhelming preclinical, clinical, and epidemiological evidence has demonstrated that cannabis produces clinically relevant dependence. Furthermore, human subjects report feelings of “high”, well-being and euphoria following the administration of THC or cannabis extracts (Ward *et al*, 1997; Haney *et al*, 1997; Hart *et al*, 2005). Therefore, cannabis produces clear subjective motivational responses and subsequently induces drug-seeking behaviour and abuse, which is the key feature common to all drugs of abuse. Several behavioural models have been used to evaluate motivational responses of drugs of abuse that could be related to their addictive properties. Although the rewarding effects of THC or other cannabinoid agonists have been difficult to reveal in animals, diverse models have effectively revealed cannabinoid-induced reward, allowing a deeper comprehension of the neurobiological mechanisms underlying cannabis addiction.

Intracranial self-stimulation

In the intracranial electric self-stimulation model, animals previously implanted with intracranial electrodes into brain-reward loci are trained to maintain an operant behaviour to obtain an electric pulse through these electrodes. A common property of most drugs of abuse is to acutely facilitate electrical stimulation of these reward-related brain areas (i.e., decreasing the threshold of the minimal current needed to promote intracranial self-stimulation), presumably due to their hedonic properties (Negus and Miller, 2014). Acute administration of THC and other cannabinoid agonists has led to contradictory results. Thus, some studies report that THC lowers intracranial self-stimulation thresholds in rats, suggesting the activation of central hedonic systems (Gardner *et al*, 1988; Lepore *et al*, 1996), while other groups have observed a lack of effect or even increased intracranial self-stimulation thresholds after the administration of diverse cannabinoid agonists (Mavrikaki *et al*, 2010; Vlachou *et al*, 2005, 2007). A possible explanation for these controversial results emerges from the biphasic nature inherent to several drugs of abuse, responsible for the opposing effects observed at low and high doses. Indeed, it has been reported that low doses of THC decreased intracranial self-stimulation thresholds, whereas high doses produced the opposite effect (Katsidoni *et al*, 2013).

Place-conditioning paradigm

An alternative measure that has been used to assess reward-related effects of cannabinoids in rodents is conditioned place preference (Tzschentke, 2007). In this paradigm, the rewarding properties of a compound are associated with the particular characteristics of a given environment. After conditioning, the animal will prefer to spend more time in the environment associated with the drug. One advantage of place-conditioning procedures is that they are sensitive not only to the rewarding but also to the aversive effects of a drug, which leads to the avoidance of the drug-associated compartment. Some evidence suggests that low doses of THC produce place preference in rats, whereas higher doses produce place avoidance (Lepore *et al*, 1995). However, in most studies THC and WIN55,212-2 produced either conditioned place aversion or no preference for either compartment (Cheer *et al*, 2000; Mallet and

Beninger, 1998; McGregor *et al*, 1996; Parker and Gillies, 1995). Pre-exposing the animals to the cannabinoid before the place-conditioning procedure has been reported to enable the development of a preference for the drug-associated compartment, probably because the first exposure is more likely to be aversive (Maldonado, 2002). Thus, mice receiving previous priming THC exposure in the home cage before the conditioning period effectively developed place preference (Valjent and Maldonado, 2000). Additionally, rats housed under environmental enrichment shifted preference towards a WIN55,212-2 associated compartment in comparison with animals housed in standard conditions (Bortolato *et al*, 2006), supporting the idea that the setting of drug use can exert a powerful modulatory influence on drug reward (Badiani, 2013).

Drug self-administration

The procedure by which animals are permitted to contingently self-administer drugs has provided a reliable method to directly evaluate the reinforcing properties of a psychoactive compound (Panlilio *et al*, 2015). Drug self-administration is therefore the preeminent animal model of drug use because it bears a close resemblance to human drug-taking and involves similar forms of conditioning and learning, with at least partial congruence of the underlying neural circuitry (Maldonado, 2002). Numerous early studies failed to demonstrate that intravenous self-administration could be maintained by THC or other cannabinoid agonists in diverse animal species, including rodents and monkeys (Carney *et al*, 1977; Harris *et al*, 1974; Mansbach *et al*, 1994; Takahashi and Singer, 1979, 1980). It has been hypothesized that the inability of THC to induce stable patterns of intake in animals is due to its pharmacokinetic properties, which may delay the onset of its psychoactive effects, and also to possible predominant aversive effects during the first exposures (Martellotta *et al*, 1998). The first evidence of cannabinoid-maintained self-administration behaviour was reported in mice, which self-infused WIN55,212-2, a synthetic cannabinoid with a shorter half-life than THC (Martellotta *et al*, 1998). However, this experiment was performed under restraining conditions and it was not until later experiments that self-administration was demonstrated in freely moving mice during repeated

daily testing (Mendizábal *et al*, 2006). In rats, reliable self-administration of WIN55,212-2 was reported in Long Evans (Fattore *et al*, 2001), and in Sprague-Dawley strains (Lecca *et al*, 2006). Another synthetic cannabinoid agonist, CP-55,940, has also been reported to sustain intracerebro-ventricular self-administration in rats (Braida *et al*, 2001b). Notably, rats are able to maintain a stable THC self-administration behaviour when the cannabinoid infusion is directly delivered into the VTA or the shell of the NAc (Zangen *et al*, 2006). However, THC self-administration by systemic route has not been yet demonstrated in rodents. In non-human primates, the first demonstration of intravenous THC self-administration was obtained in squirrel monkeys with a history of cocaine self-administration (Tanda *et al*, 2000), but subsequent work showed that THC is readily self-administered by monkeys with no prior drug experience (Justinova *et al*, 2003). Two key advantages highlight the relevance of this procedure in non-human primates: (1) doses of THC self-administered by monkeys were comparable to doses in marijuana smoke inhaled by humans (Tanda *et al*, 2000), and (2) it has enabled the development of drug-priming and cue-induced reinstatement model of relapse to THC-seeking (Justinova *et al*, 2008). However, WIN55,212-2 self-administration by rodents allows the examination of aspects of cannabis use that are difficult or impossible to study experimentally in humans or nonhuman primates, such as genetic, pharmacological or surgical manipulations. For this reason, WIN55,212-2 self-administration in mice was the behavioural model chosen to evaluate cannabinoid-induced reward in the present thesis.

2.3.6. Neurobiological mechanisms of cannabis addiction

The neurochemical processes by which cannabinoid addiction is developed are similar to those reported for other drugs of abuse. The endocannabinoid system is the major site of action for the pharmacological responses induced by cannabinoids, including the rewarding effects. Indeed, this system exerts a general modulatory effect on the reward circuitry, participating in the rewarding and addictive properties of all prototypical drugs of abuse. CB1R is abundantly expressed in diverse regions of the brain reward system, including the VTA, the NAc, the PFC and the amygdala. However, it is accepted that VTA dopaminergic neurons are unlikely to express CB1R (Julian *et al*, 2003;

Matsuda *et al*, 1993). CB1Rs present in the VTA are located on presynaptic glutamatergic and GABAergic neurons. Endocannabinoids modulate therefore the excitatory and inhibitory synaptic inputs into dopaminergic neurons of the VTA acting as a retrograde messenger (Melis *et al*, 2004; Riegel and Lupica, 2004). Thus, the activation of CB1R in the VTA, present in GABAergic interneurons and glutamatergic terminals mainly from PFC neurons, would remove these inhibitory or excitatory inputs on dopaminergic neurons respectively. The final effect on the modulation of VTA dopaminergic activity by endocannabinoids would depend on the functional balance between these GABAergic and glutamatergic inputs (Fattore *et al*, 2008; Maldonado *et al*, 2006). Accordingly, exogenous cannabinoid agonists stimulate the activity of mesencephalic dopaminergic neurons by altering this balance (Maldonado *et al*, 2006). Both THC and the synthetic cannabinoid WIN55,212-2 enhance the firing rate and bursting activity of dopaminergic neurons in the VTA (French *et al*, 1997; Gessa *et al*, 1998; Wu and French, 2000), subsequently enhancing dopamine release in terminal regions, such as the NAc and the PFC (Cheer *et al*, 2004; Chen *et al*, 1990a; Tanda *et al*, 1997), a fact that has been associated to their reinforcing properties. Moreover, the administration of WIN55,212-2 in rat VTA slices decreased GABAergic inhibitory postsynaptic currents (Szabo *et al*, 2002), supporting the idea that cannabinoids increase dopamine release by indirectly disinhibiting dopamine neurons (Lupica and Riegel, 2005). CB1Rs are key players also in the development of cannabinoid tolerance, as shown by the downregulation and uncoupling to G-protein observed in these receptors after prolonged THC exposure (Martin *et al*, 2004). Thus, rodents exposed repeatedly to cannabinoids (Fattore *et al*, 2007; Oliva *et al*, 2004) and human cannabis users (Hirvonen *et al*, 2012; Villares, 2007) present decreased CB1R expression and activity in several limbic and cortical areas (Fratta and Fattore, 2013). Yet, these cannabinoid-induced alterations in the number and function of CB1R recover within weeks upon cessation of exposure to the drug (Hirvonen *et al*, 2012), implying additional mechanisms in mediating long-lasting neurobiological changes.

Beside endocannabinoid and dopaminergic systems, other neurochemical systems have also been involved in the addictive effects of cannabinoids, including endogenous opioids, monoamines, acetylcholine, adenosine and

several neuropeptides. Among them, the role of the endogenous opioid system in the neurobiological mechanisms underlying the addictive effects of cannabinoids has been the most explored. THC-induced conditioned place preference was abolished in mice lacking mu-opioid receptors (Ghozland *et al*, 2002), and in the double knock-out for mu- and delta-opiod receptor (Castañé *et al*, 2003). In agreement, the opioid antagonist naltrexone attenuated CP-55,940-induced conditioned place-preference (Braida *et al*, 2001a) and intracerebral self-administration (Braida *et al*, 2001b) in rats, and THC self-administration in monkeys (Justinova *et al*, 2004). Accordingly, systemic administration of naloxone (Chen *et al*, 1990b; Tanda *et al*, 1997) or direct infusion of the mu-opioid antagonist naloxonazine into the VTA (Tanda *et al*, 1997) blocked the THC-induced increase of extracellular dopamine levels in the shell of the NAc, suggesting that the opioid system might control the rewarding effects of cannabinoids through a dopamine-dependent mechanism. In contrast to other opioid receptors, kappa-opioid receptors might mediate the aversive effects induced by high doses of THC and other cannabinoids. Thus, the conditioned place aversion induced by a high dose of THC was abolished in mice lacking kappa-opioid receptors (Ghozland *et al*, 2002) or pre-treated with the specific kappa-opioid antagonist nor-binaltorphamine (Zimmer *et al*, 2001). Accordingly, operant WIN55,212-2 self-administration was facilitated in mice lacking prodynorphin, the endogenous ligand of kappa-opioid receptors (Mendizábal *et al*, 2006).

2.3.7. Therapeutic strategies for cannabis dependence

Despite the high prevalence of cannabis dependence, its strong association with co-morbid mental problems and the difficulty of achieving cannabis cessation, no pharmacotherapy has been approved for cannabis dependence so far. Currently, the most successful psychotherapeutic models include cognitive behavioural, motivational enhancement, contingency management, and family-based therapies (Balter *et al*, 2014; Budney *et al*, 2007). However, nonresponse and relapse rates among patients undergoing psychosocial therapies remain high (70%), highlighting the need for the development of effective pharmacotherapies to complement these psychotherapeutic interventions (Balter *et al*, 2014). A number of pharmacological

approaches have been tested as possible experimental interventions in several laboratory and case studies in humans, which were mainly focused on promoting the initiation of abstinence, reducing withdrawal symptoms, and preventing relapse. These medications are diverse in nature, and include three major strategies for treatment: cannabinoid agonist substitution, cannabinoid antagonism, and modulation of other neurotransmitter systems.

Long-term treatment with the same agonist drug or with a cross-tolerant drug to suppress withdrawal and drug craving is among the most promising strategies to treat cannabis dependence. Dronabinol (Marinol®) is a synthetic form of THC legally marketed in many countries as an oral treatment of nausea associated with chemotherapy and for use as an appetite stimulant for AIDS or patients with cancer. Dronabinol demonstrated to be effective in suppressing cannabis withdrawal symptoms and craving in several human laboratory and case studies, particularly in combination with behavioural therapies, but it had no effect on cannabis use or relapse (Levin and Kleber, 2008; Levin *et al*, 2011, 2015; Vandrey *et al*, 2013). Nabilone (Cesamet®), a synthetic analogue of THC with similar indications than dronabinol, recently showed promise for reduction of withdrawal symptoms and relapse during laboratory trials (Haney *et al*, 2013). Nabiximols (Sativex®), an extract of cannabis containing THC and cannabidiol approved in several countries for spasticity treatment in multiple sclerosis, also attenuated cannabis withdrawal symptoms, but the effects on long-term cannabis use were not clear (Allsop *et al*, 2014). The efficacy of nabiximols in management of cannabis use disorders is currently under further investigation in a number of clinical trials. An alternative to use of direct CB1R agonists is the pharmacological inhibition of the enzymes that degrade endocannabinoids, prolonging their effects upon release and possibly relieving withdrawal similarly to a replacement therapy. Currently, the FAAH inhibitor PF-04457845 is in Phase II clinical testing for efficacy in treatment of marijuana withdrawal. PF-04457845 was well tolerated in previous clinical trials (Li *et al*, 2012), although abuse liability may be predicted since it has THC-like rewarding and reinstatement effects in squirrel monkeys (Justinova *et al*, 2014).

Drugs acting as antagonists or antagonist/inverse agonists at CB1R (such as rimonabant) represent an alternative approach to the treatment of cannabis addiction by directly blocking the rewarding effects of THC and presumably enhance relapse resistance, as observed in animal models (Justinova *et al*, 2008; Tanda *et al*, 2000). Initial findings were promising as rimonabant attenuated the subjective and physiological effects of smoked marijuana (Huestis *et al*, 2007), but withdrawal of rimonabant from clinical use due to psychiatric side effects subsequently halted further clinical development of rimonabant and other CB1R antagonists/inverse agonists (Le Foll *et al*, 2009). It has been proposed that CB1R antagonists without the inverse agonist activity presented by rimonabant might have less adverse effects and still block the effects of THC, although this possibility remains unexplored.

Based on animal research showing that mu opioid receptor antagonists block the rewarding effects of THC (Braida *et al*, 2004; Justinova *et al*, 2004), diverse human laboratory studies have investigated whether the mu opioid antagonist naltrexone can reduce the subjective effects of cannabinoids in humans. Most findings to date have been disappointing. Thus, acute naltrexone administration in cannabis users unaltered or even enhanced the subjective and physiological effects elicited by THC (Haney *et al*, 2003; Wachtel and de Wit, 2000) or smoked cannabis (Cooper and Haney, 2010; Greenwald and Stitzer, 2000). This pattern of human experimental findings suggests that naltrexone is not an effective treatment for cannabis dependence.

Numerous antidepressants have been evaluated as treatment for cannabis dependence in humans, with little success (Marshall *et al*, 2014). These include selective serotonin reuptake inhibitors (escitalopram and fluoxetine) (Cornelius *et al*, 2010; Weinstein *et al*, 2014), mixed action antidepressants (venlafaxine, nefazodone, mirtazapine and vilazodone) (Carpenter *et al*, 2009; Levin *et al*, 2013; McRae-Clark *et al*, 2016) and atypical antidepressants (bupropion) (Carpenter *et al,* 2009; Haney *et al*, 2001). The anxiolytic buspirone (McRae-Clark *et al*, 2009, 2015) and a number of mood stabilizers, such as lithium and divalproex (Johnston *et al*, 2014; Levin *et al*, 2004), also failed to reduce cannabis use and/or cannabis withdrawal symptoms. Notably, the antiepileptic gabapentin has shown promising results in attenuating withdrawal severity and reducing

cannabis use (Mason *et al*, 2012). N-acetylcysteine, a dietary supplement that might work by normalizing glutamate activity, also seems to decrease cannabis use (Gray *et al*, 2012). Several clinical trials are currently assessing the efficacy of these and other medications for treating cannabis use disorders, alone or in combination with psychotherapeutic interventions.

2.3.8. Cross-talk between orexin and endocannabinoid systems

The well-known contribution of orexins to cocaine, opioid, alcohol and nicotine addiction is not the only reason to consider their potential involvement in the addictive properties of cannabinoids. Indeed, the investigation of this possible role of the orexin system is further encouraged by emerging evidence suggesting the existence of a cross-talk between orexin and endocannabinoid systems. Their partially overlapping neuroanatomical distribution and common role as neuromodulators of several physiological and pathological processes has prompted further research to examine this putative interaction. OX1R and CB1R are able to form heteromeric complexes, as revealed by diverse studies using heterologous expression systems. Physical direct interaction between OX1R and CB1R was first suggested by electron microscopy colocalization (Hilairet *et al*, 2003), and further confirmed through single cell fluorescence resonance energy transfer imaging studies (Ellis *et al*, 2006). More recent works employing the covalently labelling of the extracellular domains of CB1R and OX1R with monitoreable peptidic tags allowed reliable tracking of these heteromers at the cell surface providing unambiguous identification of CB1R-OX1R heteromerization (Ward *et al*, 2011a, 2011b). The formation of these complexes had functional consequences at the cellular level. Hence, CB1R-OX1R presence increased the potency of orexin-A to activate the ERK signalling pathway (Hilairet *et al*, 2003), an effect reversed by the CB1R antagonist rimonabant (Ellis *et al*, 2006). The co-expression of OX1R and CB1R resulted also in coordinated trafficking of these GPCRs, which could be controlled equally by either OX1R or CB1R antagonism (Ellis *et al*, 2006). Additionally, the existence of heteromeric complexes between OX2R and CB1R has also been reported (Jäntti *et al*, 2014). Nevertheless, the biological significance of these heteromers remains unknown, and further studies are needed to

verify whether both orexin and cannabinoid receptors are expressed on the same target neurons and if they form heteromers *in vivo*.

Some studies also report that OX1R-expressing recombinant cells may release 2-AG in response to orexin-A stimulation through the activation of PLC and subsequent diacylglycerol hydrolysis by DAGL activity (Jäntti *et al*, 2013; Turunen *et al*, 2012). Recent evidence denotes that orexin signalling leads to endocannabinoid-mediated retrograde inhibition through this mechanism in diverse brain regions *in vivo* (Haj-Dahmane and Shen, 2005; Ho *et al*, 2011). Thus, electrophysiological studies report that activation of OX1R in the periaqueductal gray triggers the retrograde 2-AG-induced inhibition of GABA release, resulting in facilitated transmission through the descending antinociceptive pathway and reducing pain perception (Ho *et al*, 2011). Orexin-B has been shown to act through a similar mechanism in dorsal raphe slices, where its bath application induced depression of glutamatergic transmission to serotoninergic neurons through retrograde endocannabinoid release and activation of CB1R (Haj-Dahmane and Shen, 2005). These data support the idea that some behavioural effects of orexins might be mediated by CB1R stimulation. Indeed, the analgesic effects of an infusion of orexin-A into the rat LC before the formalin test were blocked by previous infusion of the CB1R antagonist AM251 (Kargar *et al*, 2015). Additionally, conditioned place preference induced by chemical stimulation of the LH, a process that depends on orexin signalling (Taslimi *et al*, 2011), was blocked by either intra-VTA or intra-NAc infusion of subeffective AM251 doses, suggesting that orexin transmission might signal through CB1R in the VTA and NAc to mediate this behavioural response (Fatahi *et al*, 2015; Taslimi *et al*, 2011; Yazdi *et al*, 2015). Nevertheless, the possibility that CB1R and orexin receptors produce the same response through independent mechanisms has not been ruled out in these studies.

Besides acting as a mediator of some effects exerted by orexins, the endocannabinoid system can also modulate the activity of orexin neurons. Thus, electrophysiological studies show that cannabinoid agonists reduce the activity of orexin neurons by presynaptic attenuation of glutamate release (Huang *et al*, 2007). Interestingly, a recent study shows that the balance between CB1R-expressing glutamatergic and GABAergic inputs to orexin neurons is altered in rodent models of obesity (Cristino *et al*, 2013).

In leptin knockout and diet-induced obese mice, CB1R is expressed predominantly in GABAergic inputs to orexin neurons, instead of glutamatergic, as found in healthy conditions. In addition, orexin neurons overexpress DAGL in these obese mice, with subsequent 2-AG overproduction (Cristino *et al*, 2013). These alterations could result in a retrograde inhibition of these CB1R-expressing GABAergic axon terminals, leading to disinhibition of orexin neurons and enhancing their transmission to target brain areas, which would contribute to pathological conditions related with orexin hyperfunction.

Although some research about cannabinoid modulation of orexinergic transmission has been carried out, almost no studies have examined whether orexins regulate the effects of cannabinoids or endocannabinoid function. A recent report has established a causal role for orexin-A, particularly through OX1R stimulation, in the central CB1R-mediated pressor response in conscious rats (Ibrahim and Abdel-Rahman, 2015). In this study, selective CB1R blockade had no effect on orexin-A-evoked pressor response, while the selective OX1R antagonist SB-408124 attenuated pressor response induced by either orexin-A or the cannabinoid agonist WIN55,212-2 (Ibrahim and Abdel-Rahman, 2015). Since no other studies have explored the possible modulation of endocannabinoid functions or cannabinoid-induced effects by orexins, one of our objectives was to evaluate the involvement of the orexin system in some of the prototypical acute pharmacological effects of cannabinoids, as well as in the rewarding properties of these compounds.

3. Fear and anxiety disorders

The role of the orexin system in the regulation of stress and its interaction with the HPA axis has been well established, as mentioned above. However, orexins also mobilize other components of anxiety and fear responses, such as autonomic nervous system stimulation (Johnson *et al*, 2012a). Recent reports have also linked orexins with emotional learning and memory. Importantly, the pathophysiology of several anxiety disorders is closely associated with aberrant fear memory processing, suggesting that orexins might be potential contributors to these psychiatric conditions through the modulation of aversive memories, together with the control of somatic fear and anxiety responses.

3.1. Bases of anxiety disorders

Among all psychiatric pathologies, anxiety disorders are the most prevalent in developed countries and represent high health-care utilization, resulting in an enormous economic burden for society (Bandelow and Michaelis, 2015). According to large population-based surveys, up to 30% of the general population are affected by an anxiety disorder during their lifetime (Kessler *et al*, 2012) and around 12% met the diagnostic criteria during the past 12 months (Wittchen *et al*, 2011). They are more common in women (approximately 2:1 ratio), and the highest prevalence rate is reached during midlife. Although these disorders are associated with a considerable degree of impairment, a substantial underrecognition and undertreatment of anxiety disorders has been reported. Thus, only a quarter of individuals affected with an anxiety disorder sought help from health care services (Alonso *et al*, 2004), and of those who did, a considerable proportion were not correctly diagnosed or received no treatment (Alonso *et al*, 2004; Bandelow and Michaelis, 2015). Traditional drug treatments for anxiety, such as benzodiazepines and selective serotonin reuptake inhibitors, have been shown to be beneficial for the treatment of certain anxiety disorders, but their side effects are important and a significant proportion of patients does not respond to treatment or relapses after treatment remission (Graham and Milad, 2011). Combining psychotherapeutic strategies (mainly cognitive-behavioural therapy) to pharmacotherapy has not sufficiently filled this

gap (Hofmann *et al*, 2009), revealing a clear need for an improved understanding of the neural systems underlying the pathophysiology of the diverse anxiety disorders in order to open new therapeutic approaches.

3.1.1. Concepts of anxiety and fear

Anxiety disorders include conditions that share features of excessive fear and anxiety (Farb and Ratner, 2014). Although the physical and psychological manifestations of fear and anxiety partially overlap, they are in fact two independent emotional entities differing in terms of certain key dimensions. Fear is an adaptive emotional response to a real or perceived imminent threat, that results in surges of autonomic arousal and active defensive responses (Davis *et al*, 2010; Dias *et al*, 2013). This intense and acute response normally occurs in close chronological and physical relation to the threatening stimulus, and dissipates rapidly once it is no longer present. In contrast, anxiety is a long-lasting mood state triggered by less specific and less predictable stimuli that are perceived as being potentially threatening in the future (Dias *et al*, 2013; Farb and Ratner, 2014). This perception often results in an apprehensive mood accompanied by increased arousal and vigilance in preparation for future danger. Transient, physiological fear and anxiety responses allow the individual to cope with dangerous or stressful situations, and therefore have a pivotal role in survival. However, if fear or anxiety responses to threatening stimuli are out of proportion and persist beyond the adaptive level, these emotional states can become pathological, leading to the development of an anxiety disorder (Kindt, 2014). Some anxiety disorders are particularly characterized by abnormal fear processing, whereas others are defined by more general pathological anxiety states (see below). Moreover, individuals suffering from these pathological conditions often attempt to reduce the level of fear or anxiety through pervasive avoidance behaviours, leading to progressive social and physical isolation and promoting co-occurrence of other mental disorders (Farb and Ratner, 2014).

3.1.2. Anxiety disorders: a wide spectrum of mental conditions

Anxiety disorders include a broad range of recognized clinical conditions, each of which is unique with respect to the intensity and duration of the fear or anxiety experienced as well as the type of stimuli that can induce it. Thus, although anxiety disorders tend to be highly comorbid with each other, they can be differentiated by close examination of the types of situations that are feared or avoided and the content of the associated thoughts or beliefs. Their classification and diagnostic criteria have considerably changed throughout the successive editions of DSM (Table 4), but the distinctive features of the main recognized anxiety disorders have remained invariable and are described below (APA, 2013).

Table 4. Classification of anxiety disorders according to the 4th and the 5th editions of DSM.

Disorder name	Previous (DSM-4) classification in	Current (DSM-5) classification in
	Shifted categories	
Panic disorder (PD)	Anxiety disorders (as linked diagnostic entities: PD without agoraphobia, agoraphobia with/without PD)	Anxiety disorders (as independent diagnostic entities)
Agoraphobia		
Obsessive-compulsive disorder	Anxiety disorders	Obsessive-compulsive and related disorders
Posttraumatic stress disorder (PTSD)	Anxiety disorders	Trauma- and stressor-related disorders
Acute stress disorder	Anxiety disorders	Trauma- and stressor-related disorders
Separation anxiety disorder	Disorders usually diagnosed in childhood and adolescence	Anxiety disorders
Selective mutism	Disorders usually diagnosed in childhood and adolescence	Anxiety disorders
	Unchanged categories (common DSM-4 and DSM-5 anxiety disorders)	
Specific phobia		Substance/medication-induced anxiety disorder
Social anxiety disorder (social phobia)		Anxiety disorder due to another medical condition
Generalized anxiety disorder		Unspecified anxiety disorder

In panic disorder, the individual experiences recurrent panic attacks, defined as abrupt surges of intense fear that reach a peak within minutes, accompanied by somatic symptoms, such as shortness of breath, chest pain, light-headedness, and sweating. In panic disorder, panic attacks are usually unexpected and are referred to as "uncued", since they occur in the absence of any identifiable source of danger. Because these symptoms occur spontaneously and without warning, persons with panic disorder are persistently worried about the consequences of having a panic attack and use to change their behaviour in maladaptive ways, typically avoiding situations that their past experience suggests can trigger the onset of an attack.

In contrast to panic disorder, specific phobias are featured by "cued" fear attacks, defined as those in which the sufferer experiences debilitating symptoms in response to a clearly defined object or situation. In this case, exposure to the phobic stimulus produces an immediate onset of increased anxiety or fear to a degree that is persistent and out of proportion to the actual risk posed, and is severe enough to interfere with the individual's ability to function normally in social or occupational settings. According to DSM-5, specific phobias are classified depending on the fear-triggering stimulus: animal (e.g., spiders, dogs), natural environment (e.g., heights, storms), blood-injection-injury (e.g., needles, invasive medical procedures), situational (e.g., airplanes, enclosed spaces) and other (e.g., costumed characters, loud sounds).

Agoraphobia is a type of phobia that is classified under its own category in the DSM-5, mainly due to the amount of patients presenting this specific disorder in comparison to other phobias. Individuals with agoraphobia are fearful or anxious about two or more of the following situations: using public transportation; being in open spaces; being in enclosed places; standing in line or being in a crowd; or being outside of the home alone in other situations. The individual fears these situations because of thoughts that escape might be difficult or help might not be available in the event of developing panic-like or other incapacitating symptoms. It is noteworthy that two thirds of patients suffering from panic disorder present comorbid agoraphobia because of their distorted belief that they will need emergency help when having a panic attack, and they

subsequently start to avoid situations in which getting medical help would be impossible or embarrassing (Möller *et al*, 2015).

In social phobia (or social anxiety disorder), another highly prevalent phobia with its own diagnostic criteria in the DSM-5, the individual is anxious about or avoidant of social interactions that involve the possibility of being examined or judged. These include social situations in which the individual may be observed eating or drinking, or require meeting unfamiliar people or performing in front of others. The cognitive ideation is of being negatively evaluated by others, by being embarrassed, humiliated, or rejected.

Generalized anxiety disorder is characterized by persistent and excessive anxiety and worry in response to an overload of stimuli related to diverse domains, including routine life events as job performance, health of family members, or simply being on time for appointments. In addition, the affected individuals experience physical symptoms, including restlessness, fatigue, concentration problems, irritability, and muscle tension. Individuals with generalized anxiety disorder can be differentiated from those with non-pathological anxiety by two main criteria: (1) they find it difficult to control their worrying, and (2) this perseverative thought process interferes with their ability to function in social and occupational settings. The onset of this anxiety disorder is typically uncertain and is often not associated with a readily identifiable precipitating event (Farb and Ratner, 2014).

Some anxiety disorders are triggered upon exposure to a traumatic or stressful event, such as being in an automobile accident or witnessing a murder. Psychological distress following exposure to catastrophic or aversive events is quite variable. In most of the cases, symptoms can be well understood within an anxiety- or fear-based context. However, since some individuals also exhibit phenotypes different from fear or anxiety, including anhedonic, aggressive, or even dissociative symptoms, the aforementioned disorders have been grouped under a separate category in DSM-5. Acute stress disorder is an example enclosed within this diagnostic category, and is characterized by symptoms that occur during the first month after exposure to the traumatic event that evokes intense fear and/or a sense of helplessness. Symptoms of acute stress disorder

include hypervigilance, exaggerated startle response, avoidance of places and events that remind of the traumatic event, and recurrent intrusive memories or dreams about the event. Although severe enough to interfere with activities of daily living, these symptoms typically resolve spontaneously within about 4 weeks after the event. By contrast, posttraumatic stress disorder (PTSD) is characterized by similar symptoms that persist for more than 1 month and can last for years after exposure to the stressful event.

Finally, obsessive-compulsive disorder and other related clinical conditions are characterized by the presence of obsessions and/or compulsions. Obsessions are recurrent and persistent thoughts, urges, or images that are experienced as intrusive and unwanted, whereas compulsions are repetitive behaviours or mental acts that an individual feels driven to perform in response to an obsession or according to rules that must be applied rigidly. In obsessive-compulsive disorder, the anxiety experienced by the individual is about what will happen if he or she does not recurrently perform these particular behaviours or compulsions (e.g., repetitive hand washing). In DSM-5, obsessive-compulsive and related disorders have been removed from the anxiety disorders chapter to one of their own, since these medical conditions have more differences than similarities in symptomatology, aetiology, genetics, neurobiology and treatment response (Stein *et al*, 2011).

3.1.3. Current pharmacotherapies against pathological anxiety

An extensive variety of pharmacological therapeutic strategies has been employed during the last decades to relief the symptoms of the above described medical conditions (Farb and Ratner, 2014). This diversity arises from the fact that fear and anxiety are processes controlled by multiple neurotransmitters and neuromodulators, and dysfunction of each of these systems appears to contribute to the pathophysiology of anxiety disorders (Millan, 2003). The main anxiolytic strategies include:

- Positive modulation of GABAergic transmission. There is general agreement that GABA-mediated inhibitory neurotransmission plays a particularly important role in the modulation of emotional responses to fear-inducing stimuli, and exerts an inhibitory effect on the release of the

main neurotransmitters involved in stress responses and anxiety (Davis and Myers, 2002; Kalueff and Nutt, 2007). Allosteric potentiation of GABAergic inhibitory neurotransmission by benzodiazepines have been central to anxiolytic pharmacology for decades (Chan and Farb, 1985; Millan, 2003), but they present side effects such as drowsiness, impaired motor coordination, amnesia, and abuse potential (Farb and Ratner, 2014).

- Enhancement of serotoninergic transmission. The role of serotonin in pathological anxiety has become increasingly well established (Farb and Ratner, 2014), and agents that inhibit the reuptake of this neurotransmitter represent a possible treatment for a range of anxiety disorders (Graeff and Zangrossi, 2010). The mechanisms by which these compounds improve the symptoms of certain anxiety disorders are complex and beyond the scope of this thesis, but it seems that their antidepressant effects may contribute to symptom relief, since individuals affected by anxiety disorders with a depressive component, such as PTSD, respond better to selective serotonin reuptake inhibitors (Farb and Ratner, 2014).

- Modulation of noradrenergic transmission. Noradrenergic transmission, stemming from the LC, activates the autonomous nervous system eliciting diverse somatic effects such as increase in the cardiorespiratory function, and stimulates certain regions of the corticolimbic system enhancing attention and vigilance (Tanaka *et al*, 2000). Anxiety disorders are associated with hyperfunction of noradrenergic transmission, which can lead to hyperarousal states (Bremner *et al*, 1996; Southwick *et al*, 1999). Thus, substances like clonidine, an agonist for α2-adrenergic presynaptic autoreceptors (hence, inhibitor of noradrenaline release), present anxiolytic effects (Charney and Redmond, 1983) and present potential therapeutic use in hypervigilance conditions such as PTSD (Belkin and Schwartz, 2015). Likewise, propranolol, a β-adrenergic receptor antagonist, is employed to reduce the vegetative symptoms of anxiety (Emilien and Maloteaux, 1998).

- Inhibition of general neurotransmission. Since anxiety has been associated with a neuronal hyperexcitability in response to stress, reducing the synaptic release of neurotransmitters in within the CNS

appears to be an effective strategy. Pregabalin, one of the newer anxiolytic medications, achieves its anxiolytic effect by binding to the $\alpha_2\delta$ subunit of the P/Q-type of voltage-gated calcium channel, resulting in reduced neurotransmitter release and controlled propagation of neurotransmission (Micó and Prieto, 2012). A review of available evidence indicates that pregabalin is a well-tolerated and consistently effective treatment for generalized anxiety disorder (Baldwin *et al*, 2015), and recent studies report also its efficacy in social anxiety disorder (Kawalec *et al*, 2015).

- Modulation of glutamatergic transmission. The inhibition of glutamatergic activity represents a similar strategy, attempting to restore the balance between excitatory and inhibitory neurotransmission (Simon and Gorman, 2006). Memantine, a NMDA receptor antagonist, is able to PTSD, social phobia and generalized anxiety disorder (Battista *et al*, 2007; Schwartz *et al*, 2012). The inhibitor of glutamate release riluzole is also able to relief generalized anxiety (Pittenger *et al*, 2008). On the other hand, promoting glutamatergic transmission during exposure therapy facilitates learning not to fear the salient stimulus. Thus, the partial NMDA receptor agonist D-cycloserine has enhanced recovery of phobias and PTSD among patients undergoing behavioural exposure therapy (Difede *et al*, 2014; Ressler *et al*, 2004).

3.2. Physiological and pathological fear

Although an important part of fear responses are innately programmed and well conserved across species, the process of learning is crucial to successfully overcome new potential dangers and be able to identify predictors of danger (Kindt, 2014). Given that associative fear memory lies at the root of fear and anxiety disorders, understanding the neurobiological mechanisms that mediate long-term storage, retrieval and weakening of fear memories is of great interest.

3.2.1. Fear responses and their modelling in animals

The encounter with real or potential threats involves the activation of a variety of alarm or fear responses, including autonomic, hormonal or behavioural (Figure 12). These responses define the state of fear that

emerges from the activation of the neural defence circuits and its reflexive autonomic and somatic outputs (Lang *et al*, 2000). Fear responses are highly conserved across mammals: most of the typical signs and symptoms observed and measured in rodent models of fear are also reported in humans experiencing fear or panic attacks (Dias *et al*, 2013). Importantly, fear responses are plastic, which allows to adjust the responses to the situational demands either by increasing or by decreasing them (Riebe *et al*, 2012). There exists a broad variety of animal models that enable the study of the processes underlying this bidirectional modulation of fear responses. This section summarizes the main paradigms and read-outs employed to study fear according to its nature or adaptation: innate fear, learning of fear, and relief of fear.

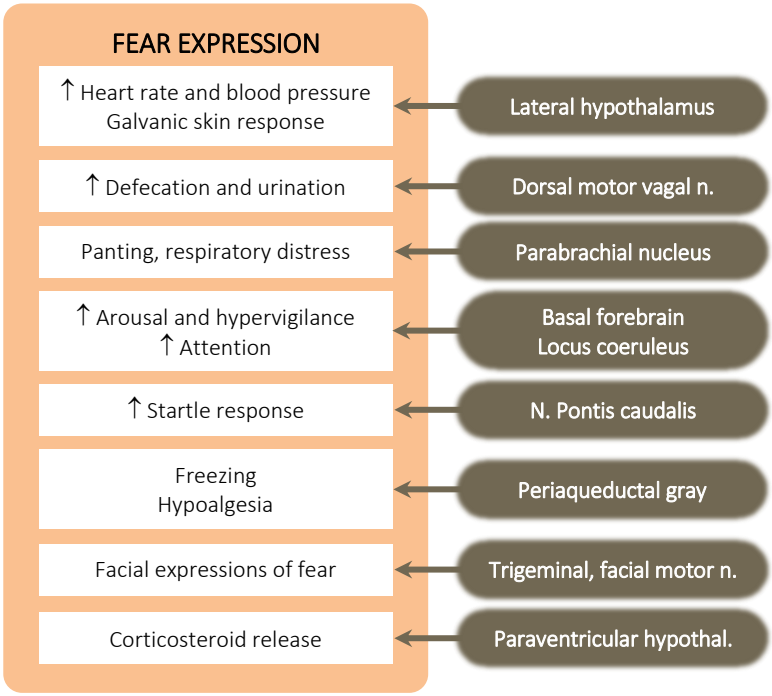


Figure 12. Characteristic behavioural and somatic responses to fearful stimuli. These fear responses are highly conserved across mammals: they are typically displayed by rodents, and most of them are also observed in humans experiencing fear or panic attacks. Putative brain regions responsible for triggering each of these fear responses are also displayed. (Adapted from Dias *et al*, 2013, and Lang *et al*, 2000).

3.2.1.1. Innate fear: unconditioned models

The study of unconditioned or ethological responses to different forms of external threats is a logical extension and simulation, in laboratory conditions, of what occurs in nature (innate fear/avoidance). Examples of paradigms exploring this kind of responses are:

Predator encounter-based models. Predator/prey interactions model fear and anxiety depending on whether or not a threat is immediate or anticipated, and this threat level can be manipulated by changing the type of stimulus used (Campos *et al*, 2013). Although humans do not innately fear 'predators' *per se*, this rodent model is thought to be equivalent to the urgent desire to escape a situation or place where extreme anxiety or panic occurs (Ganella and Kim, 2014). In rodents, exposure to a live cat or to its odour elicits diverse defensive behaviours (including fight, autonomic activation, and ultrasonic vocalizations) among which freezing is the most typical response used as a measure of fear (Campos *et al*, 2013). "Freezing", a behavioural response operationally defined as the absence of movement other than breathing, is a species-specific defence response exhibited by rodents and is probably the most widely used measure of fear in rodent research, especially in conditioned fear paradigms (see below).

Unconditioned startle responses. This measure of unlearned fear is elicited by the presentation of an intense, unexpected stimulus (usually a noise, a flash of light or a tactile stimulus), and it is a response conserved across many species (Lang *et al*, 2000). In humans, eye-blink is taken as the index of the startle response, while in rodents the whole body jump is usually taken as the magnitude of startle (Davis *et al*, 1993). The startle reflex is increased by fear and aversive states (Grillon *et al*, 1997). Thus, rodents exhibit a potentiated acoustic startle response when tested in a brightly illuminated environment, whereas humans exhibit a larger startle response when tested in the dark.

Approach-avoidance conflict. The basic premise of these models is the set of behavioural responses induced by exposure to a new environment (e.g., brightly lit or elevated areas), which simultaneously evokes fear and curiosity, creating a typical approach/avoidance conflict (Campos *et al*, 2013). These behavioural models are employed to quantify trait anxiety

and anxiety generalization, rather than acute fear episodes, and include paradigms such as the elevated plus maze, the light-dark box, and the open field (Campos *et al*, 2013).

3.2.1.2. Learned fear: conditioned models

Alterations in fear learning and cognitive defects form an important facet of the clinical manifestation of anxiety disorders, including inappropriate processing of potentially threatening stimuli in generalized anxiety disorder, panic disorder and phobias, as well as the long-term salience of traumatic memories in PTSD (Riebe *et al*, 2012). Aversive test conditions often lead to the formation of aversive memories, resulting in increased fear responses during subsequent encounters (LeDoux, 2000). This may include both associative and non-associative processes (Riebe *et al*, 2012). Associative conditioning involves stimulus-stimulus associations (i.e., classical Pavlovian conditioning) or stimulus-response associations (i.e., operant conditioning).

Classical (Pavlovian) conditioning. Pavlovian fear conditioning involves an associative learning process in which a neutral conditional stimulus (CS), such as a light, tone, or setting, is repeatedly paired with an aversive unconditional stimulus (US), such as an electric foot-shock. After the repeated pairings, re-exposure to the CS alone will elicit autonomic and behavioural fear-conditioned responses on its own, such as freezing as mentioned earlier. Fear-potentiated startle is another commonly used index of fear, and is the increase in startle response when the fear-associated stimulus (e.g., CS associated with shock) is present compared with when it is absent. Other widely used measures include avoidance behaviour and changes in heart rate or arterial pressure (Ganella and Kim, 2014). According to the nature of the CS, fear conditioning can be referred to as "cued", where the CS is an elemental cue (e.g., a tone, a light, an odour), or "contextual", where the CS entails the integration of different elemental cues into a general environmental representation (Riebe *et al*, 2012) (Figure 13A). Pharmacological studies of conditioned fear typically involve three distinctive phases: (1) the acquisition phase, which refers to the actual associative phase during which paired presentations of the CS and the US are given to the animal; (2) the consolidation phase, immediately after the acquisition phase and it is the

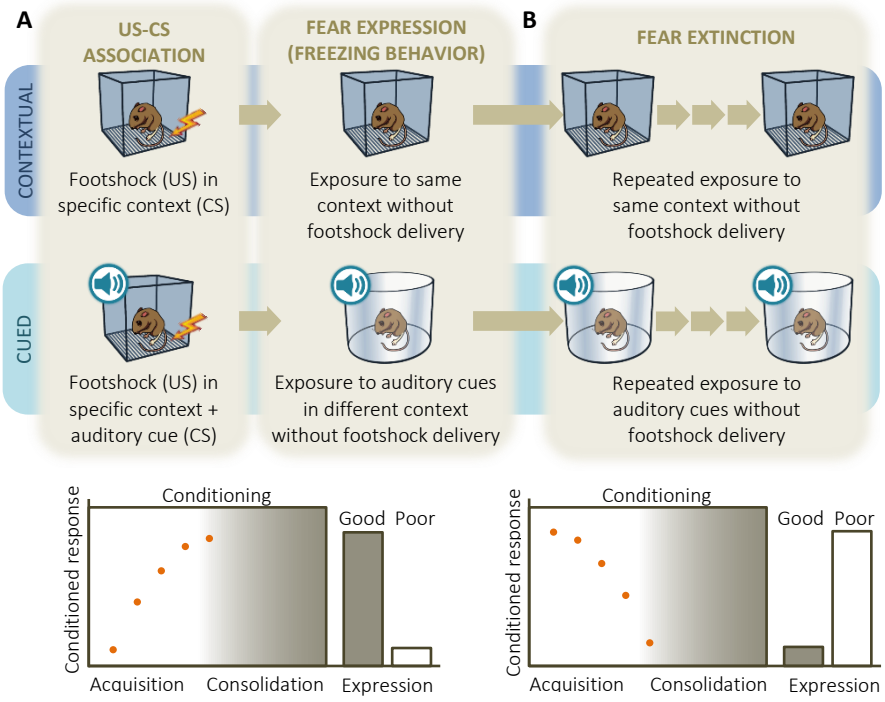


Figure 13. Contextual and cued fear conditioning (FC) and extinction paradigms. A. During conditioning, a particularly neutral conditioned stimulus (CS), usually a chamber in contextual FC (upper diagram) or a tone in cued FC (lower diagram), is presented together with an aversive unconditioned stimulus (US), habitually an electrical footshock. As a consequence of this US-CS association, a new exposure to the CS in absence of the US evokes an evaluable conditioned response (i.e., freezing). **B**. Repeated exposure to the CS in absence of the US progressively leads to fear extinction, expressed as the decrease of the freezing response and weakening of the US-CS association. Both conditioning and extinction processes comprise the phases of: acquisition (during the learning session), consolidation (after the learning session), and expression (during the test session). (Adapted from Ganella and Kim, 2014).

time-window required for the working/short-term memory to become a stable, long-term memory; and (3) the expression or retrieval phase, when the animal is tested with CS-alone trials to examine whether it can elicit a fear conditioned response (Figure 13A) (VanElzakker *et al*, 2014).

Operant conditioning. In contrast to classical conditioning, operant conditioning leads to the formation of stimulus-response associations, which enable the animals to avoid the threatening stimulus in the future. According to the nature of the conditioned response, these models are referred as: (1) passive avoidance paradigm, whereby the animals learn to suppress escape behaviours in order to avoid a foot-shock; or (2) active

avoidance paradigm, whereby they execute directed escape responses during an acoustic stimulus that signals the subsequent occurrence of a foot-shock. It is worth to mention that passive avoidance can be "contaminated" by freezing responses caused by contextual conditioning (Riebe *et al*, 2012).

3.2.1.3. Relief of fear

The ability to recognise the absence and/or disappearance of a threat is a crucial component of fear processing, necessary to successfully adapt the individual's responses to the new unthreatening situation. There are a number of processes and interventions that may ultimately result in a lasting decrease in fear responses. Some of them require reactivation of the original fear memory (e.g., extinction training, disruption of reconsolidation) and/or exposure to the threatening situations (safety learning), whereas others do not (e.g., erasure, forgetting) (Riebe *et al*, 2012).

Forgetting and erasure. Forgetting describes the decrease in fear with the mere passage of time. It seems that there might be some contribution of forgetting to the maintenance of contextual fear in animal experiments, but there is little evidence for forgetting of conditioned fear with elemental cues (Riebe *et al*, 2012). Memory erasure describes the reversal of those changes in the fear circuitry that had been caused by fear conditioning, and may result from pharmacological manipulation of particular components involved in the maintenance of fear (Maren, 2011). However, its clinical application is currently discarded, since it is practically impossible to selectively erase a particular memory trace, which would cause a general amnesia.

Extinction training. Extinction training procedures are based on repeated exposures to the conditioned cue/context in the absence of the predicted punishment or threat with the goal of reducing fear responses from exposure to exposure (Figure 13B). Extinction has received considerable attention over the past decade because of its theoretical importance and its obvious clinical implications for the treatment of various anxiety disorders (Myers and Davis, 2002; Quirk and Mueller, 2008). Additionally, conditioned fear and extinction models are believed to have substantial

face (phenotypical), construct (aetiological) and predictive (treatment responsiveness) validity (Milad *et al*, 2014; VanElzakker *et al*, 2014). Indeed, the current cognitive-behavioural treatments for anxiety disorders in humans largely involve some type of exposure therapies that rely on the process of extinction (Hofmann, 2008). Early theoretical models suggested that this decreased response to the CS after extinction was due to the 'unlearning' or 'erasure' of the original CS–US association, but currently it is widely believed that the decrease in the conditioned response after extinction is mainly due to new inhibitory learning (i.e., the formation of a new memory trace, which inhibits the expression of the original fear memory) (Bouton, 2002; Herry *et al*, 2008). This process of extinction is fundamentally context-dependent: once both a CS-US (conditioning) and a CS-noUS (extinction) representation exist, the response relevant to CS-noUS is only expressed in the context in which this new association was learned (Bouton, 2004; Bouton *et al*, 2006).

Safety learning. This form of long-term fear relief states that stimuli that remain explicitly unpaired with an electric foot-shock (or another aversive stimulus) are not seen as neutral controls and they gain the status of safety signals (Riebe *et al*, 2012). Although few studies have employed this procedure, some reports highlight its relevance by revealing that safety learning hindered fear conditioning with the same stimuli, reduced contextual fear and caused antidepressant-like effects (Christianson *et al*, 2012; Pollak *et al*, 2008).

Reconsolidation disruption. When a CS is presented after conditioning, the fear memory is reactivated, enters a labile state (Nader *et al*, 2000; Sara, 2000), and opens two possibilities: (1) reconsolidation (strengthening of the fear memory), or (2) reconsolidation disruption, using either a pharmacologic agent or immediate extinction within the lability window, and leading to a long-term reduction in fear responses (Diergaarde *et al*, 2008). Importantly, this procedure has shown to cause long-lasting fear relief in a study with human volunteers (Schiller *et al*, 2010).

3.2.2. Neurobiological substrates of fear learning and memory

There is substantial knowledge about the brain regions and neurotransmitter systems that are involved in fear processing. Most of

our understanding about the neurobiology of fear processing arises from studies using the classical fear conditioning and extinction paradigms (LeDoux 2000, Tovote 2015). Therefore, this section focuses on the neuroanatomical and neurochemical substrates of fear learning inferred from studies based on these behavioural procedures and complemented with electrophysiology, pharmacological, optogenetic and functional imaging approaches.

3.2.2.1. Neuroanatomy of fear conditioning

The brain structures mediating fear conditioning are well characterized in rodents and humans (Maren and Quirk, 2004). A remarkable number of studies have identified the amygdala, the hippocampus, and the medial PFC as the classical regions of the fear neural circuit, although other brain areas are also involved in the modulation of fear (Maren *et al*, 2013). The amygdala is the leading brain structure controlling fear learning and expression, as it receives and integrates information about conditioned and unconditioned stimuli, and subsequently promotes and orchestrates a series of fear reactions through projections to the behavioural, autonomic, and endocrine response systems located in the brainstem (LeDoux, 2000) (Figure 14). The amygdalar complex is composed of

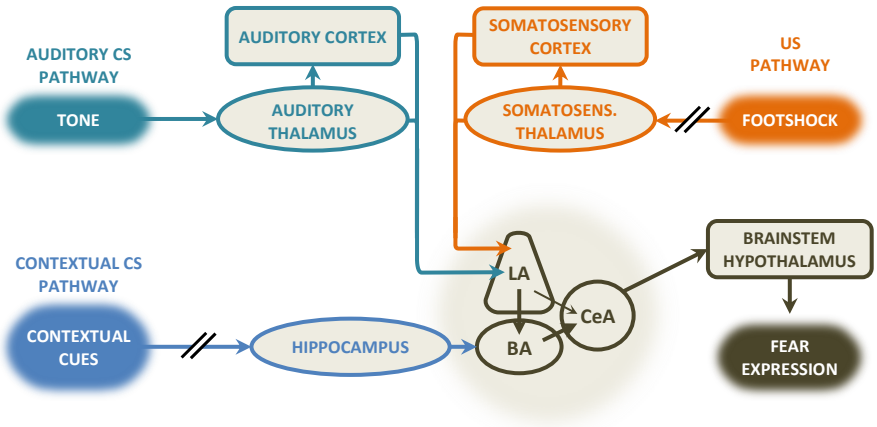


Figure 14. Fear conditioning pathways. The auditory conditioned stimulus (CS) and somatosensory (pain) unconditioned stimulus (US) converge in the lateral amygdala (LA) via thalamic and cortical inputs. Contextual cues require integration by the hippocampus and reach the basal and accessory basal (BA) amygdala. The central amygdala (CeA) controls the expression of fear responses. (Adapted from LeDoux, 2000, 2007).

diverse nuclei, each of which executes a particular function in fear processing (LeDoux, 2007). The basolateral amygdala (BLA), including lateral, basal, and accessory basal nuclei, is the major gateway for information regarding the CS and the US, and represents an important site for the plastic changes underlying CS-US association (Herry and Johansen, 2014). Thus, auditory and other sensory inputs from elementary cues come from both thalamic and cortical projections to the lateral amygdala, and contextual information is transmitted to the basal and accessory basal amygdala from the hippocampus, which is responsible for assembling the different components of the contextual cues into a single representation of the context (LeDoux, 2000, 2007; Rudy, 2009). US information travels from primary afferent nociceptors and the spinal dorsal horn to the lateral amygdala, probably through diverse supraspinal processing stations, such as the periaqueductal gray and the thalamus (Herry and Johansen, 2014). On the other hand, the central nucleus of the amygdala represents its main output region responsible for the expression of fear responses through projections to downstream structures in the brainstem and the hypothalamus, involved in the physiological and behavioural fear readouts detailed earlier (Figure 14) (Lang *et al*, 2000; Orsini and Maren, 2012). Importantly, the sensory information received by the lateral amygdala is further processed through intra-amygdala connections before reaching the central nucleus, since direct connections between these two nuclei are rather scarce (LeDoux, 2007). The formation of fear memories also requires the participation of the prelimbic division of the PFC (dorsal anterior cingulate in humans), which has excitatory projections to the BLA and constitutes a key pathway for the cortical modulation of fear (Figure 15A) (Sierra-Mercado *et al*, 2011). Inactivation of the prelimbic cortex reduces the expression of cued and contextual fear (Corcoran and Quirk, 2007), while microstimulation of this area leads to the opposite effect (Vidal-Gonzalez *et al*, 2006), supporting the assertion that the prelimbic cortex regulates fear expression by activating the amygdala.

Fear extinction recruits similar brain regions to those involved in the expression of aversive memories, but instead of the prelimbic PFC, fear extinction engages the infralimbic subdivision of the PFC (ventromedial PFC in humans) (Figure 15B) (Sierra-Mercado *et al*, 2011). Indeed,

prelimbic and infralimbic subdivisions of the PFC have opposing influences on fear processes, since the infralimbic cortex is required for fear extinction but not fear acquisition, and prelimbic stimulation even impairs fear extinction (Sierra-Mercado *et al*, 2011; Vidal-Gonzalez *et al*, 2006).

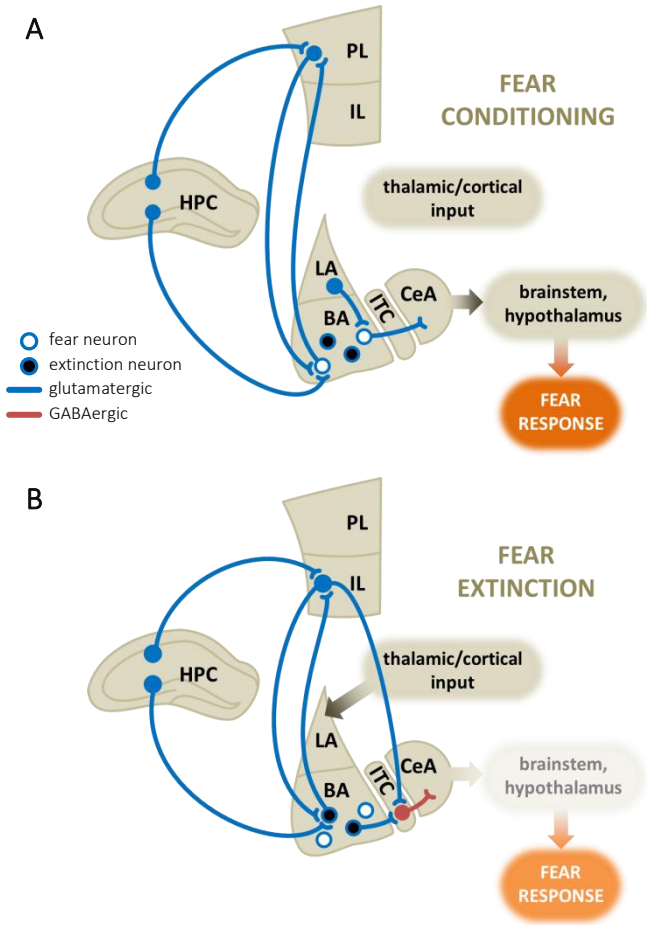


Figure 15. Neural circuitry of fear expression and extinction. A. During fear expression thalamic and cortical inputs conveying information about the CS and the US arrive first at the LA, which excites the CeA through stimulation of a particular neuronal subpopulation in the BA known as "fear neurons", eliciting fear responses via successive projections to brainstem and hypothalamic sites. The BA receives also excitatory input from PL cortex, thereby promoting the expression of conditioned fear. B. During fear extinction the IL cortex excites the GABAergic ITC neurons, either directly or through activation of the "extinction neurons" subpopulation in the BA. ITC neurons inhibit the CeA, thereby inhibiting conditioned fear and promoting extinction. In addition, the HPC modulates both PL and IL as well as amygdalar activity, integrating contextual information processing. BA, basal amygdala; CeA, central amygdala; HPC, hippocampus; IL, infralimbic prefrontal cortex; ITC, intercallated cells; LA, lateral amygdala; PL, prelimbic prefrontal cortex.

Interestingly, the basal amygdala is involved in both the formation and the extinction of fear memory, and two distinct populations of projecting neurons have been identified in this brain structure: (1) the so-called "fear neurons", which are active during fear conditioning and show reciprocal connections with the prelimbic PFC; and (2) the "extinction neurons", which respond during fear extinction and are connected bidirectionally with the infralimbic cortex (Herry *et al*, 2008; Senn *et al*, 2014). Thus, infralimbic PFC neurons project to extinction neurons within the basal amygdala, which in turn activate another amygdalar nuclei referred as intercalated neurons (Royer and Paré, 2002; Royer *et al*, 1999). These interneurons are GABAergic and project to the central amygdala, inhibiting its activity and supressing the expression of fear. Intercalated neurons putatively receive glutamatergic input also from the infralimbic PFC, further promoting the inhibition of the fear response (Figure 15A) (Myers and Davis, 2007). The hippocampus, which has robust reciprocal connections with the basal amygdala (Pitkänen *et al*, 2000), also appears to be essential for fear extinction (Myers and Davis, 2007). As previously mentioned, the new association CS-noUS that is formed during extinction is linked to the context where extinction training takes place. During exposure of the animal to the extinction context, the hippocampus has been proposed to activate the infralimbic cortex and certain inhibitory neurons in the BLA, thus inhibiting output neurons in the central amygdala and preventing fear expression. If the hippocampal function has been hindered, or if the animal is exposed to a context different from the one where extinction was performed, the aforementioned hippocampal projections do not prevent fear expression induced by the former existing CS-US association (Quirk and Mueller, 2008).

3.2.2.2. Molecular modulators of fear learning and memory

A considerable number of studies have revealed the molecular underpinnings involved in fear processing, particularly those implicated in the formation and extinction of aversive memories. This section is focused in those neurochemical modulators the manipulation of which would allow an improvement of fear extinction, due to their potential clinical relevance.

- Endocannabinoid system. A considerable body of evidence points to a crucial role of the endocannabinoid system in the regulation of the behavioural domains of acquired fear, anxiety and stress-coping (Lutz *et al*, 2015). This neuromodulatory system appears to be particularly involved in the extinction of aversive memories, but not in the acquisition of these memories (Moreira and Wotjak, 2010). Thus, CB1R knockout mice, as well as mice treated with the CB1R antagonist rimonabant, exhibited impaired extinction of auditory fear, but unaltered acquisition and consolidation memory (Marsicano *et al*, 2002). Analysis of contextual fear revealed essentially the same findings (Riebe *et al*, 2012). Accordingly, administration of the CB1R agonist WIN55,212-2, as well as increasing the endocannabinoid tone by using endocannabinoid reuptake inhibitors or FAAH inhibitors, facilitated the extinction of conditioned fear (Chhatwal *et al*, 2005; Pamplona *et al*, 2006). It should be noted that the degree of aversion to negative stimulus must exceed a certain threshold before the endocannabinoid system is activated to mitigate the fear response, indicating that it might serve as a protection system that prevents the emergence of exaggerated responses to fear (Kamprath *et al*, 2009; Moreira and Wotjak, 2010). Interestingly, recent studies have revealed that endocannabinoids can also act as promoters of fear expression when acting in certain brain regions or neuronal populations. Thus, increased 2-AG levels promote the expression of conditioned fear (Llorente-Berzal *et al*, 2015) through CB1R located on GABAergic neurons. Another study reports that deletion of CB1R from cholinergic neurons within the medial habenula reduces fear-conditioned freezing (Soria-Gómez *et al*, 2015), further supporting that endocannabinoids can also enhance fear expression depending on their localization.

- BDNF/TrkB pathway. The brain-derived neurotrophic factor (BDNF) is one of the most studied neurotrophins involved in synaptic plasticity processes. BDNF primarily produces its effects by interacting with the tropomyosin-related kinase B receptor (TrkB). BDNF/TrkB signalling appears to be crucial for the formation of fear memories, since BDNF+/- mice present deficits in the acquisition of contextual fear conditioning (Korte *et al*, 1995; Patterson *et al*, 1996), whereas TrkB-overexpressing mice present the opposite phenotype (Koponen *et al*, 2004). Interestingly, conditional knockout mice with a BDNF gene deletion in the hippocampus

exhibited deficits in extinction of cued fear-conditioning, suggesting a role for this neurotrophin also in extinction of aversive memories (Heldt *et al*, 2007). Supporting this theory, it has been observed that infusion of recombinant BDNF in the infralimbic cortex enhances cue-dependent extinction in rats, even without extinction training (Peters *et al*, 2010). Furthermore, a single systemic dose of 7,8-dihydroxyflavone, a TrkB agonist, enhances both fear acquisition and extinction in naïve mice, and more importantly, rescues a deficit in fear extinction in a potential mouse model of PTSD, were mice are subjected to 2-hour immobilization 6 days before fear conditioning and extinction (Andero *et al*, 2011). Interestingly, other neurotrophic factors have also been linked with fear-related conditions. Indeed, mice overexpressing the neurotrophin-3 receptor, TrkC, present increased anxiety- and panic-like reaction, increased contextual fear conditioning and impaired fear extinction, and have been suggested to be an engineered murine model of panic disorder (Santos *et al*, 2015).

- Glucocorticoid system. As previously stated, stressful and fear-evoking stimuli activate the HPA with subsequent release of glucocorticoids from the adrenal cortex. These hormones can cross the blood-brain barrier and bind to glucocorticoid receptors, present in relevant areas within the fear circuitry (Bentz *et al*, 2010). Diverse reports revealed that glucocorticoids enhance the consolidation of both cued and contextual fear conditioning, while blockade of glucocorticoid receptors hinders these behavioural responses (Rodrigues *et al*, 2009). Interestingly, this glucocorticoid-induced facilitation in the consolidation process has also been observed during extinction training in animals and humans (Blundell *et al*, 2011; Ninomiya *et al*, 2010; Yang *et al*, 2006). This fact has highlighted the potential use of glucocorticoids as extinction enhancer in combination with exposure-based therapies (de Bitencourt *et al*, 2013). Indeed, PTSD patients have reduced cortisol levels (Yehuda *et al*, 2004), and daily administration of cortisol reduces symptoms related to the traumatic memories of these patients (Aerni *et al*, 2004; de Quervain *et al*, 2011).

3.2.3. Aberrant fear extinction in anxiety disorders

It is widely accepted that anxiety disorders are maintained as a result of a failure to appropriately extinguish fear. Several studies using laboratory

tasks have consistently demonstrated that clinically anxious populations exhibit deficits in fear extinction (Graham and Milad, 2011). Thus, individuals with panic disorder exhibited larger skin conductance responses during extinction training, despite showing no differences from healthy subjects in conditioned responses during or following conditioning (Michael *et al*, 2007). Impaired extinction has also consistently been reported in individuals with PTSD when compared to trauma-exposed subjects without PTSD (Blechert *et al*, 2007; Peri *et al*, 2000). Interestingly, this abnormal behavioural response appeared to be positively correlated with symptom severity in these individuals (Norrholm *et al*, 2011). Considerable evidence indicates that clinically anxious populations also exhibit alterations in the neural circuitry that mediates normal extinction. Recent studies using imaging techniques report that individuals with low anxiety levels showed a positive coupling between amygdala resting state activity and ventromedial (infralimbic in rodents) PFC activity, and negative coupling in those with high anxiety levels (Kim and Whalen, 2009; Kim *et al*, 2011). These results suggest that dysfunctions in connectivity between these two brain regions may mediate susceptibility to anxiety disorders. Moreover, individuals with PTSD and specific phobia presented diminished ventromedial PFC activity when exposed to trauma reminders or specific fear-related stimuli, respectively, in comparison to control subjects (Hermann *et al*, 2009; Shin *et al*, 2005). Compared with healthy subjects, individuals with PTSD also showed decreased resting activity in the ventromedial PFC and the hippocampus, but heightened activity in the dorsal anterior cingulate (prelimbic PFC in rodents), during fear extinction (Bremner *et al*, 2005; Milad *et al*, 2009). These findings, together with the positive correlation observed across all participants between the magnitude of extinction and activity in the ventromedial PFC and hippocampus (Milad *et al*, 2009), suggest that hyperactivity in the dorsal anterior cingulate and hypoactivity in the ventromedial PFC may contribute to the impairment of extinction observed in PTSD.

3.3. Role of orexins in fear and anxiety

3.3.1. Orexins as mediators of physiological anxiety and fear processing

As mentioned above, several recent reports support a role of the orexin system in the regulation of fear, anxiety and stress responses (Johnson *et al*, 2012a). This physiological function is consistent with the neuroanatomical location of orexin neurons and their projections to brain areas involved in the modulation of motivation and emotion. Indeed, the perifornical and dorsomedial regions of the hypothalamus, where a great portion of orexin-expressing neurons is located, have been largely described as the part of the limbic circuit that controls "fight-or-flight" reactions in response to an imminent threat (Hess and Akert, 1955; Johnson *et al*, 2012a). Stimulation of these hypothalamic areas through different approaches triggers a series of fear-associated responses, such as tachycardia, hyperventilation and increased locomotion in animals (Anderson and DiMicco, 1990; Duan *et al*, 1994), and self-reported panic in humans (Wilent *et al*, 2010). These findings led to these regions of the hypothalamus being referred to as the hypothalamic "defence area". In addition, orexin neurons are interconnected to brain areas that mobilize different components of anxiety and fear responses (Nambu *et al*, 1999; Peyron *et al*, 1998), such as: (1) stress and arousal systems, including the monoaminergic systems in the LC, dorsal raphe and tuberomammillary nucleus; (2) limbic and related brain regions (e.g., BNST, lateral septum, amygdala, PFC, cingulate cortex); (3) autonomic and respiratory sites, including adrenergic rostroventrolateral medulla, periaqueductal gray, and parabrachial nucleus; and (4) stress hormone sites for sympathetic activation, like the PVN. Moreover, a number of electrophysiological studies demonstrate that orexins directly excite neurons within diverse nuclei responsible for autonomic, respiratory and cardiovascular responses to stressful and panic-inducing stimuli (Johnson *et al*, 2012a). Overall, orexin neurons are ideally positioned to integrate a variety of emotional and stress-associated sensory signals and mobilize an adaptive behavioural and physiological response to deal with the threat and restore homeostasis.

Substantial evidence has demonstrated the role of orexins as modulators of physiological responses to emotional and stressful stimuli. The cardiovascular and locomotor responses elicited by exposure to an intruder mouse are diminished in mice lacking orexins (Kayaba *et al*, 2003). Similarly, cardiovascular responses to air-jet stress were reduced in orexin/ataxin-3 mice with postnatal loss of orexin neurons (Zhang *et al*, 2006). It appears that the excitatory input from the amygdala and BNST to orexin neurons contributes to these responses, since disinhibition of either the BNST or the amygdala by $GABA_A$ receptor antagonist microinjections elicited cardiorespiratory excitation in wildtype mice but not in orexin/ataxin-3 mice (Zhang *et al*, 2009). Conversely, orexin-A microinjections either into the BNST (Lungwitz *et al*, 2012) or the amygdala (Avolio *et al*, 2011) promoted anxiogenic-like states in rodents, denoting the collaboration between these regions and orexin neurons in processing emotional responses. The PVT may also be an important relay site for anxiety and arousal mobilization by orexin neurons (Boutrel *et al*, 2010). Indeed, orexins excite PVT neurons that project to the cortex *in vitro,* which may be important for arousal (Huang *et al*, 2006), and *in vivo* microinjections of orexin-A and -B in the PVT promoted increases anxiety and vigilance associated behaviours in the elevated plus maze (Li *et al*, 2010), further supporting the contribution of orexins to anxiogenic-like states.

In correlation with these findings in animals, individuals with narcolepsy (and therefore lacking orexin neurons) show reduced autonomic responses to emotional stimuli, especially aversive ones (Tucci *et al*, 2003), whereas they have a normal cardiovascular response to physical homeostatic challenges (e.g., Valsalva manoeuvre and cold pressor test). This suggests that orexin regulates the sympathetic nervous system primarily in response to salient emotional cues or contexts. Moreover, an fMRI study revealed that narcoleptic patients show reduced amygdala activity and low functional coupling between the amygdala and medial PFC during aversive conditioning, as well as abnormal emotional learning (Ponz *et al*, 2010). In agreement, patients with narcolepsy failed to exhibit startle potentiation during unpleasant stimuli (Khatami *et al*, 2007), supporting the idea that human narcolepsy should be considered not only as a sleep–wake disorder, but also as a condition with reduced reactivity

of the aversive motivational system responsible for negative or unpleasant emotions. Interestingly, a recent study in subjects suffering from treatment-resistant temporal lobe epilepsy that were implanted with microdialysis probes showed an increase of orexin-A levels in the amygdala during positive emotions, social interaction, and anger (Blouin *et al*, 2013). Since these behaviours often induce cataplexy in narcoleptic patients, it has been suggested that activation of orexin neurons by the limbic system maintains wakefulness during emotional arousal by conveying various emotional stimuli to orexin neurons. Overall, these findings denote that correct emotional processing requires a preserved orexinergic function, which seems to be especially relevant in relationship with the amygdala.

Recent evidence from rodent models has demonstrated that orexins are also involved in fear memory formation and consolidation. Rats receiving acute oral treatment with the DORA almorexant displayed reduced fear-potentiated startle responses in the presence of a fear-associated stimulus (Steiner *et al*, 2012). In agreement, mice lacking OX1R displayed reduced freezing and reduced lateral amygdala activation (expression of the immediate-early gene Zif268) in response to both cued and contextual fear stimuli (Soya *et al*, 2013). Interestingly, it seems that orexinergic projections to the LC contribute to the establishment of fear memory. Noradrenergic neurons of the LC have been previously involved in emotional memory formation, since the activity of these neurons increases after fear-conditioning (Ishida *et al*, 2002), and noradrenaline release in the lateral amygdala increased with presentation of stressful stimuli in rats (Galvez 1996). Re-expression of OX1R in noradrenergic LC neurons of OX1R knockout mice by using an adeno-associated virus vector restored both freezing behaviour and lateral amygdala activation after cued fear conditioning testing (Soya *et al*, 2013). A similar involvement of OX1R in the LC in amygdala-dependent fear learning was revealed by pharmacological and optogenetic approaches (Sears *et al*, 2013). In this study, microinjection of the OX1R antagonist SB-334867 within the LC before conditioning impaired cue-induced fear memory formation, whereas optical stimulation of orexin fibres in the LC during conditioning enhanced this behavioural response (Sears *et al*, 2013). These findings suggest that OX1R signalling within the LC is important during the learning

phase of fear memory formation. In contrast, mice lacking OX2R showed reduced freezing behaviour in a contextual fear test, but normal freezing in a cued fear paradigm (Soya *et al*, 2013). In agreement, central infusion of the OX2R antagonist TCS-OX2-29 unaltered freezing responses in a cue-induced fear-conditioning paradigm (Sears *et al*, 2013), further supporting the dissociation between OX1R and OX2R signalling in the formation of cued versus contextual fear memories. These observations, together with the abnormal emotional learning reported in narcoleptic patients, support the crucial role of orexins as mediators of emotional memory formation during fearful situations.

3.3.2. Orexins as contributors to anxiety disorders

As mentioned above, impaired fear processing is a key feature of a range of anxiety disorders. Given the role of the orexin system in the modulation of the behavioural expression of fear responses, orexin-related targets have emerged as promising opportunities for treating anxiety disorders. Hyperactivity of the orexin system has been suggested to be a critical contributor to the pathological maintenance of arousal and anxiety, and it might increase vulnerability to relapse to panic episodes in pre-disposed individuals (Johnson *et al*, 2012a). In agreement, humans with panic disorder have elevated levels of orexin-A in the CSF compared with control subjects (Johnson *et al*, 2010). Interestingly, orexin reduction is a possible mechanism for the anti-panic effects of some antidepressant drugs, since this reduction has been observed after chronic sertraline treatment, which exerts effective anti-panic actions, but not after bupropion treatment, which results inefficient in treating panic disorder (Salomon 2003). Moreover, the anxiolytic effects of benzodiazepines could be partially mediated by inhibition of orexin neurons, since diazepam reduces c-Fos expression in these cells (Panhelainen and Korpi, 2012). By contrast, a clinical study showed that CSF and plasma orexin-A levels are reduced in combat veterans with chronic PTSD, and negatively correlated with PTSD severity (Strawn *et al*, 2010). These unexpected results apparently contradict the association between a hyperactive orexin system and a higher vulnerability to develop maladaptive responses to fear events. However, possible depressive symptoms could be masking these findings, since depression has been associated with low

orexin levels in CSF even in the presence of comorbid anxiety (Johnson *et al*, 2010). Furthermore, abnormal fear conditioning represents a trademark in the pathophysiology of PTSD (Mahan and Ressler, 2012), and the role of orexins in the modulation of this neurobiological process suggests that they might be relevant contributors to this medical condition. Another factor involved in PTSD development is the presence of generalized avoidance of situations with low similarity to the originally fear-associated context after experiencing a traumatic episode, which predicts a higher probability of developing this anxiety disorder (van der Velden *et al*, 2006). Interestingly, a recent study in rats has reported a connection between the orexin system and this maladaptive response (Viviani *et al*, 2015). Chronic dual orexin receptor blockade with almorexant reduced both the development and persistence of generalized avoidance in situations with low similarity to the initial footshock context (Viviani *et al*, 2015), suggesting that targeting the orexin system might be a possible preventive strategy for PTSD and other anxiety disorders presenting generalized avoidance.

Panic disorder has also been robustly linked with orexins in animal models. Anatomical and pharmacological approaches in rodents have revealed that the DMH/PFA, among other brain regions, is activated during panic attacks (Johnson *et al*, 2008). Moreover, orexin knockout mice display attenuated cardiovascular and behavioural defence responses to emotional stress after disinhibition of the DMH/PFA (Kayaba *et al*, 2003). Based on these observations, a rat model of panic disorder involving chronic disinhibition of DMH/PFA and putative increase in orexin activity has been developed and validated over the past years (Johnson and Shekhar, 2012). In this model, chronic reduction of GABA synthesis in the rat DMH/PFA using l-allylglycine produces anxiety-like states, as measured by social interaction and elevated plus maze tests, and enhanced vulnerability to panic-like responses (i.e., cardiorespiratory stimulation and flight-like locomotion) following intravenous infusions of lactate (Johnson and Shekhar, 2006). Further studies confirmed that chronically removing inhibitory GABAergic tone in the DMH/PFA to produce panic-prone rats selectively increased local orexin neuronal activity that was correlated with anxiety states (Johnson *et al*, 2010). Interestingly, systemic administration of the OX1R antagonist SB-334867,

as well as local silencing of the orexin precursor gene in the PFA region through siRNA injection, attenuated anxiety-like behaviour and cardiorespiratory responses induced by the lactate challenge in panic-prone rats (Johnson *et al*, 2010). These findings, together with the increased orexin-A CSF levels observed in patients suffering from panic anxiety, suggest that OX1R antagonists may provide an alternative therapeutic approach for the treatment of panic disorder.

Orexin modulation of the pH/CO_2 balance appears to be closely related to its role in panic disorder. Changes in CO_2 and acid concentration may trigger panic attacks after a challenge test by increasing autonomic symptoms and promoting behavioural arousal (Esquivel *et al*, 2009). Notably, recent evidence demonstrated that orexin neurons are highly sensitive to local changes in CO_2/H^+ (Nattie and Li, 2012), and this chemosensitivity is involved in mobilizing panic and anxiety-associated responses to panicogenic doses of hypercapnia (>10%) (Johnson *et al*, 2012a). Thus, exposure to hypercapnic (20%) normoxic gas increased c-Fos expression in orexin neurons and promoted anxiety-like behaviour in rats, an effect prevented by systemic injection of the OX1R antagonist SB-334867 (Johnson *et al*, 2012b). These findings may be relevant for subjects presenting episodes of hypercapnia, such as patients with chronic obstructive pulmonary disease (COPD), bronchitis or asthma. These conditions show significant comorbidity with severe anxiety and increased risk of panic attacks, and the management of these symptoms might be challenging because potent anxiolytics might also slow down respiratory drive, which is needed to expulse CO_2 during hypercapnic episodes. A human study has reported that orexin-A plasma levels are dramatically increased in patients with COPD (Zhu *et al*, 2011). Consistent with this, chronic exposure to cigarette smoke tripled hypothalamic orexin-A expression in a rodent model of COPD (Liu *et al*, 2010). Thus, the orexin system may also be an important target in future management of panic and anxiety in patients suffering from COPD and other hypercapnic conditions.

In summary, hyperfunction of the orexin system might be a critical contributor to the pathological maintenance of anxiety and fear through diverse mechanisms, including mobilization of autonomic, hormonal and behavioural responses to internal or external threats. On the other hand,

the inability to properly extinguish fear is an important component of several anxiety disorders. Although the role of orexins as mediators of aversive memory formation during fearful situations has been recently revealed, the possible modulation of fear memory extinction by orexins has not been explored yet, and constitutes one of the main objectives of the present thesis.

OBJECTIVES

General objective

Growing data support a role for the orexin system in drug addiction and anxiety disorders. Therefore, the main goals of this thesis are to investigate the role of orexin transmission in the addictive properties of cannabinoids and to elucidate the contribution of orexins to emotional memory processing.

Specific objectives

1. To study the participation of the orexin system in the classical behavioural responses induced by acute THC administration in mice (Article 1).
2. To evaluate the involvement of the orexin transmission in the reinforcing properties of cannabinoids (Article 2).
3. To assess the neuroanatomical basis of the possible role of orexins in cannabinoid-induced reward (Article 2).
4. To compile and analyse the existing evidence pointing to a molecular, functional and behavioural cross-talk between orexin and endocannabinoid systems (Article 3).
5. To elucidate the role of orexins in the consolidation and extinction of contextual and cued fear memories (Article 4).
6. To evaluate the neuroanatomical substrates underlying the possible role of orexins in fear memory extinction (Article 4 and Supplementary Results).
7. To investigate the molecular pathways involved in the possible modulation of fear extinction by orexins (Supplementary Results).
8. To update and summarize the role of the orexin system in fear processing and its contribution to anxiety disorders (Article 5).

RESULTS

ARTICLE 1

Involvement of the orexin/hypocretin system in the pharmacological effects induced by Δ^9-tetrahydrocannabinol

África Flores, Marina Julià-Hernández,
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Br J Pharmacol (2016) 173(8):1381-1392

Flores Á, Julià-Hernández M, Maldonado R, Berrendero F. Involvement of the orexin/hypocretin system in the pharmacological effects induced by Δ 9 -tetrahydrocannabinol. Br J Pharmacol. 2016 Apr;173(8):1381–92. DOI: 10.1111/bph.13440

ARTICLE 2

The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward

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Rafael Maldonado and Fernando Berrendero

Biol Psychiatry (2014) 75(6):499-507

Flores Á, Maldonado R, Berrendero F. The Hypocretin/ Orexin Receptor-1 as a Novel Target to Modulate Cannabinoid Reward. Biol Psychiatry. 2014 Mar 15;75(6):499–507. DOI: 10.1016/j.biopsych.2013.06.012

ARTICLE 3

Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far

África Flores,
Rafael Maldonado and Fernando Berrendero

Front Neurosci. (2013) 7:256. *Review*

Flores A, Maldonado R, Berrendero F. Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far. Front Neurosci. 2013 Dec 20;7:256.
DOI: 10.3389/fnins.2013.00256

ARTICLE 4

The hypocretin/orexin system mediates the extinction of fear memories

África Flores, Victòria Valls-Comamala,
Giulia Costa, Rocío Saravia,
Rafael Maldonado and Fernando Berrendero

Neuropsychopharmacology (2014) 39(12): 2732-2741

Flores Á, Valls-Comamala V, Costa G, Saravia R, Maldonado R, Berrendero F. The Hypocretin/Orexin System Mediates the Extinction of Fear Memories. Neuropsychopharmacology. 2014 Nov 16;39(12):2732–41.
DOI: 10.1038/npp.2014.146

ARTICLE 5

Orexins and fear: implications for the treatment of anxiety disorders

África Flores, Rocío Saravia,
Rafael Maldonado and Fernando Berrendero

Trends Neurosci (2015) 38(9): 550-559. *Review*

Flores Á, Saravia R, Maldonado R, Berrendero F. Orexins and fear: implications for the treatment of anxiety disorders. Trends Neurosci. 2015 Sep;38(9):550–9. DOI: 10.1016/j.tins.2015.06.005

DISCUSSION

The orexin system was originally thought to specifically mediate feeding and promote wakefulness, but it is now clear that this neuropeptide system participates in a wide range of behavioural and physiological processes that might reflect an important function for orexins. Orexin transmission seems to be particularly relevant under situations of high motivational relevance, such as during physiological need states, exposure to threats or reward opportunities. Therefore, homeostatic signals of physiological disequilibrium and/or exposure to important environmental stimuli appear to recruit the orexinergic function, contributing to translate these motivational (either appetitive or aversive) or challenging states into adaptive behavioural and physiological responses (e.g., enhanced arousal, suppressed sleep, increased reward-seeking, activation of the stress-hormonal system, etc.). Importantly, a growing evidence supports that the orexin system contributes also to neuropsychiatric conditions associated with dysfunctional processing of these incentive or defiant situations (Chen *et al*, 2015). Thus, it has been revealed that orexins contribute to the processes underlying the development of drug addiction and modulate the addictive properties of cocaine, opioids, alcohol and nicotine (Plaza-Zabala *et al*, 2012b). Nevertheless, the potential participation of these neuropeptides in the addictive properties of cannabinoids has not been yet elucidated. On the other hand, several recent reports support a role for orexins in the regulation of fear- and anxiety-driven responses, and hyperfunction of the orexin system has been suggested to increase vulnerability to develop certain anxiety disorders (Johnson *et al*, 2012a). However, whether the orexin system regulates aberrant processing of emotional memory, a pivotal contributor to some anxiety disorders, has remained largely unexplored.

By the use of behavioural and biochemical studies, mainly through pharmacological and genetic manipulations of the orexin system, we investigated the possible influence of orexin transmission in: (1) the behavioural effects of cannabinoids, including the acute pharmacological and reinforcing effects, and (2) the consolidation and extinction of fear memories. The results described in this thesis are the first to report the

modulation of THC-induced hypothermic, antinociceptive and anxiolytic-like effects by OX2R, as well as the specific involvement of OX1R signalling in the reinforcing effects of the synthetic cannabinoid WIN55,212-2. Moreover, we confirmed the role of orexins in the consolidation of fear memories, and we established a crucial function of orexin transmission through OX1R stimulation in the extinction of aversive memories, which seems to influence the communication between the amygdala and the medial PFC.

1. Participation of the orexin system in cannabinoid-induced effects

Cannabis derivatives have become the most consumed illicit drug all over the world and constitute a major health concern in developed countries, primarily among the young population. Due to its detrimental effects on brain development, early onset of cannabis consumption has been associated with increased risk for developing psychiatric disorders. Other undesirably consequences of cannabis use include cognitive impairment, anxiogenic effects, alterations in motor coordination and cannabis dependence (Hall, 2015). Cannabis is mostly taken for its relaxing, euphorigenic and hedonic properties, but its therapeutic potential as analgesic, anti-anorectic, anti-emetic and neuroprotective agent have raised even more the interest of the community for this illicit drug. Although the potential medicinal use of cannabis should certainly be explored, the public perception of cannabis use as a harmful practice is weakening, a fact that might possibly increase even more its consumption and the subsequent cannabis dependence prevalence rates (Cook *et al*, 2015). Therefore, one of the major challenges in cannabinoid research consists on identifying possible mechanisms to dissociate the therapeutic actions of cannabis from its detrimental consequences, among which cannabis addiction represents a pivotal concern.

THC, the main psychoactive constituent of cannabis preparations, produces a wide spectrum of central and peripheral actions, including antinociception, hypothermia, hypolocomotion, catalepsy, memory

disruption, rewarding effects and emotional responses (Pertwee, 2008). The central pharmacological effects of THC are mainly mediated by CB1R, as evidenced by the lack of THC-induced antinociceptive, hypolocomotor and hypothermic effects of THC in CB1R knockout mice (Ledent *et al*, 1999). The pharmacological responses induced by THC and other cannabinoids are modulated by several heterologous systems different from the endocannabinoid system, including dopamine (Sami *et al*, 2015), endogenous opioids (Robledo *et al*, 2008), monoamines (Viñals *et al*, 2015), GABA (Radhakrishnan *et al*, 2015), glutamate (Castaldo *et al*, 2010), adenosine (Justinová *et al*, 2014) and diverse neuropeptides (Verty *et al*, 2004). A growing body of evidence suggests that the orexin system may also be potentially involved in cannabinoid-induced effects, since several recent studies point to the existence of a bidirectional crosstalk between orexinergic and endocannabinoid systems (Article 3). CB1R is able to form heteromeric complexes with both OX1R and OX2R in diverse heterologous expression systems. Formation of CB1R-OX1R heteromers resulted in coordinated trafficking of these GPCRs as well as alterations in downstream signalling upon co-stimulation of both receptors, including cross-antagonism phenomena (i.e., the ability of an antagonist of one receptor to interfere the signalling of the partner receptor) (Ellis *et al*, 2006; Jäntti *et al*, 2014). Moreover, activation of the OX1R leads to 2-AG production, suggesting that endocannabinoids could contribute to some orexin effects (Ho *et al*, 2011; Turunen *et al*, 2012). Indeed, the endocannabinoid system has been reported to modulate some physiological functions of orexins, such as food intake (Crespo *et al*, 2008; Cristino *et al*, 2013) and nociception (Cristino *et al*, 2016; Ho *et al*, 2011; Lee *et al*, 2016). However, few studies have evaluated so far whether orexins participate in the pharmacological effects of cannabinoids. In this thesis, we demonstrate that orexins contribute to several behavioural responses induced by cannabinoids, and reveal that OX1R and OX2R signalling specifically modulate distinct effects of these compounds.

Arousal and wakefulness promotion are considered the trademark of orexin peptides. Central administration of orexin-A increases arousal and

locomotor activity in rodents (Hagan *et al*, 1999; Mang *et al*, 2012), an effect reversed by both OX1R and OX2R antagonism (Mang *et al*, 2012; Plaza-Zabala *et al*, 2010). It is not surprising therefore that THC-induced hypolocomotion remained unaltered in mice lacking the prepro-orexin gene or pre-treated with OX1R/OX2R antagonists (Article 1), suggesting that orexin transmission does not contribute to this effect of THC. Interestingly, prepro-orexin knockout mice displayed a lower basal locomotor activity in comparison with wildtype animals, in concordance with previous observations (Anaclet *et al*, 2009; España *et al*, 2007; Plaza-Zabala *et al*, 2010). However, hypolocomotion induced by prepro-orexin gene deletion and by THC administration did not appear to be additive, suggesting that either both effects might partially share the same mechanism, or hypolocomotion reached a ceiling effect under these particular conditions (Article 1).

In contrast to the effects in locomotion, we observed that THC-induced hypothermia was abolished in prepro-orexin knockout mice (Article 1). Orexins seem to contribute to the hypothermic effects of THC through OX2R signalling, since the OX2R antagonist TCS-OX2-29 reduced THC-induced hypothermia, whereas OX1R pharmacological blockade or gene deletion unaltered this cannabinoid response (Article 1). Orexin transmission has been generally associated with increased thermogenesis, although contradictory results have been reported (Messina *et al*, 2014). Thus, central infusion of orexin-A induces a generalized activation of the sympathetic nervous system, accompanied by an increase in brown adipose tissue and colonic temperatures (Monda *et al*, 2004, 2007). Accordingly, rats with genetic ablation of orexin neurons show reduced thermogenesis in response to a threatening event, although baseline levels of body temperature were unaltered in these orexin-ablated animals (Mohammed *et al*, 2014). However, orexin-A administration at lower doses than those used in the above-mentioned reports produces hypothermia and hypometabolism in rats (Balaskó *et al*, 1999; Jászberényi *et al*, 2002), an anabolic response that seems to be favoured in moderately cool environments (Székely *et al*, 2002). Interestingly, prepro-

orexin knockout mice showed decreased THC-induced c-Fos expression in the medial preoptic area (Article 1), an important thermoregulatory region (McKinley *et al*, 2015) containing a dense projection of orexin-positive nerve terminals (Peyron *et al*, 1998) and high density of OX2R mRNA (Marcus *et al*, 2001). Therefore, our results suggest that cannabinoids could partially induce their hypothermic effects by promoting orexin signalling in the medial preoptic area. However, other brain regions may also be responsible for THC-induced hypothermia mediated by orexins, since classic studies demonstrate that THC induces a hypothermic response also in rats with lesioned preoptic regions (Schmeling and Hosko, 1976).

Orexins are antinociceptive peptides, as revealed in different assays for thermal, mechanical and chemical stimuli (Mobarakeh *et al*, 2005) and in mouse models of inflammatory (Bingham *et al*, 2001) and trigeminovascular pain (Holland *et al*, 2005). The antinociceptive effects of orexins, similar to those exerted by THC, take place at both spinal and supraspinal level (Chiou *et al*, 2010). Our study reveals that orexin transmission contributes to the antinociceptive effects of THC at the supraspinal level. Thus, antinociceptive effects of THC in the hot plate test –mainly evaluating the supraspinal component of nociception– were reduced in prepro-orexin knockout animals as well as in TCS-OX2-29-pre-treated mice (Article 1). In contrast, THC-induced antinociceptive responses in the tail immersion test –mainly measuring the spinal component of pain perception– remained unaltered in prepro-orexin and OX1R knockout mice and in animals pre-treated with the OX1R or OX2R antagonists (Article 1). These results suggest that orexins contribute to the supraspinal antinociceptive effects of THC through OX2R signalling. The antinociceptive effects of orexins have been classically associated with OX1R activation (Bingham *et al*, 2001; Jeong and Holden, 2009; Yamamoto *et al*, 2003a, 2003b). However, the increasing availability of selective OX2R antagonists has favoured the identification of OX2R signalling as a contributor to orexin-induced antinociception in diverse forms of pain (Azhdari-Zarmehri *et al*, 2013; Ezzatpanah *et al*, 2016; Yazdi

et al, 2016). Indeed, the antinociceptive effects of orexins are blocked by local OX2R antagonism in diverse brain regions important for pain regulation, such as the periaqueductal gray (Lee *et al*, 2016), the NAc and the VTA (Azhdari-Zarmehri *et al*, 2013). Nevertheless, THC-induced increase in c-Fos expression remained unaltered in the periaqueductal gray and the NAc of prepro-orexin knockout animals (Article 1), although this fact does not exclude their participation in this antinociceptive interaction. Notably, prepro-orexin knockout mice displayed lower THC-induced activation of the central amygdala (Article 1), a region that receives ascending nociceptive signals, has efferent projections to areas involved in pain modulation and is crucial in controlling pain threshold and its emotional component (Palazzo *et al*, 2011). However, OX2R expression in the central amygdala is relatively low, and further studies would be needed to clarify whether this reduced activation following THC administration in prepro-orexin knockout mice is due to absent OX2R signalling. On the other hand, several studies have reported that the endocannabinoid 2-AG mediates the antinociceptive effects induced by orexins at diverse supraspinal regions, including the periaqueductal gray (Cristino *et al*, 2016; Ho *et al*, 2011; Lee *et al*, 2016) and the LC (Kargar *et al*, 2015). Taken together, these data indicate that bidirectional interactions may underlie the antinociceptive properties of cannabinoids and orexins, which brings to mind the phenomenon of cross-antagonism observed between CB1R and OXRs in cellular expression systems (Ellis *et al*, 2006; Ward *et al*, 2011b).

THC effects on anxiety are biphasic since low doses of THC induce anxiolytic-like effects and high doses anxiogenic-like responses (Berrendero and Maldonado, 2002; Valjent *et al*, 2002). Orexin peptides, particularly through OX1R, promote anxiety-like effects in diverse behavioural models of anxiety and stimulate the HPA axis in response to stressful stimuli (Suzuki *et al*, 2005; Winsky-Sommerer *et al*, 2004). Moreover, they contribute to the anxiogenic-like effects of other drugs of abuse, such as nicotine (Plaza-Zabala *et al*, 2010). However, our results obtained in the elevated plus maze revealed that the anxiogenic-like

effects of THC are independent from orexin transmission, since the reduction of time spent in the open arms following THC administration was unaffected by pharmacologic or genetic manipulations of the orexin system (Article 1). Importantly, the basal anxiety levels of mice pre-treated with OXR antagonists or prepro-orexin and OX1R knockout mice were similar to those displayed by control groups (Article 1), in agreement with previous results indicating that these manipulations primarily reduce anxiety-like states induced by diverse challenging stimuli, but not basal anxiety levels (Plaza-Zabala *et al*, 2010; Rodgers *et al*, 2013; Staples and Cornish, 2014). Surprisingly, THC-induced anxiolytic-like effects were abolished in prepro-orexin knockout animals and in mice pre-treated with the OX2R antagonist TCS-OX2-29 (Article 1). Although few reports have examined the role of OX2R in anxiety, a recent study has established an association between OX2R and anxiolysis (Arendt *et al*, 2014). In this study, knocking down the OX2R, but not the OX1R, in the BLA of mice increased anxiety as measured by reduced social preference and reduced time spent in the central area of an open field (Arendt *et al*, 2014). In agreement, our results show that the OX2R mediates the anxiolytic-like effects of THC, which could have important therapeutic implications. However, we could not evaluate the role played by orexins in the basolateral amygdala because c-Fos expression was almost absent in this brain region both after THC exposure and in basal conditions, as previously reported (Valjent *et al*, 2002). Indeed, neuroimaging studies in humans report that THC exposure significantly reduced lateral amygdala reactivity to social signals of threat (Phan *et al*, 2008), supporting that THC-induced effects in this region cannot be detected in terms of increased activation.

One of the most characteristic detrimental effects of THC is memory impairment, which can be evaluated in the novel object recognition test (Puighermanal *et al*, 2009). We confirmed that the amnesic-like effects induced by THC in this behavioural task were not mediated by orexins, since these effects remained unaffected in prepro-orexin and OX1R knockout mice and in mice pre-treated with OX1R or OX2R antagonists

(Article 1). Indeed, these neuropeptides have been reported to facilitate cognition, especially attention and certain types of learning and memory (Wheeler *et al*, 2014). They contribute to hippocampus-dependent social recognition memory (Yang *et al*, 2013), and improve the cognitive performance of sleep-deprived nonhuman primates (Deadwyler *et al*, 2007). However, dual orexin receptor antagonism does not affect object recognition memory in rats at doses that affected sleep (Uslaner *et al*, 2013). It seems that orexins primarily promote learning and cognition that involves emotionally or motivationally relevant stimuli, but not (or in a lesser extent) non-emotional forms of learning, including the one involved in the novel object recognition task. Thus, both orexin receptors have been involved in fear conditioning (Sears *et al*, 2013; Soya *et al*, 2013; Article 4) and passive avoidance tasks (Akbari *et al*, 2008; Palotai *et al*, 2014). Probably for this reason, contradictory results have been reported regarding the effects of orexin antagonists in spatial memory using the Morris water maze, since this paradigm might involve certain levels of stress (Akbari *et al*, 2007; Dietrich and Jenck, 2010). In agreement, we observed that prepro-orexin and OX1R knockout mice, as well as those pre-treated with OX1R or OX2R antagonists, displayed normal basal memory parameters in the object recognition test (Article 1), as confirmed in posterior observations (Article 4).

The possible mechanism by which orexins contribute to THC-induced hypothermia, supraspinal antinociception and anxiolytic-like effects remains to be elucidated. However, the finding that prepro-orexin knockout mice show lower THC-induced c-Fos expression in certain brain regions, including the central amygdala, the lateral septum and the medial preoptic area might provide some clues with this regard. Although these areas participate in THC-induced effects modulated by orexin transmission (i.e., antinociception, anxiolysis and hypothermia), their lower THC-induced reactivity might be related to other non-evaluated pharmacological effects of this cannabinoid. Importantly, this reduced local reactivity to THC exposure in prepro-orexin knockout mice was unlikely to be a consequence of altered CB1R expression levels in these

animals, since immunoblot analyses revealed that the total amount of these receptors remains unaffected in the brain regions examined (Article 1). Other alterations in CB1R signalling, such as intracellular receptor location or modified G-protein binding, cannot be discarded in these mutant mice.

Similarly to other drugs of abuse, cannabis derivatives and synthetic cannabinoids enhance the activity of the reward system, producing euphoria and drug-taking behaviour. These rewarding effects are crucial during the initial phases that potentially drive the subject to develop addictive behaviour, fomenting repeated drug-use through the processes of conditioning and learning. Drug self-administration is the preeminent animal model of drug use because it is a valid and reliable predictor of whether a drug will have rewarding effects in humans, involving similar forms of conditioning and learning and presumably similar neurobiological mechanisms. THC self-administration by systemic route has not been yet demonstrated in rodents. Therefore, we employed the operant self-administration of the synthetic cannabinoid WIN55,212-2, a reliable model in which mice are able to maintain stable patterns of cannabinoid intake (Mendizábal *et al*, 2006). In this paradigm, orexin transmission demonstrated to modulate the reinforcing effects of this synthetic cannabinoid through OX1R signalling (Article 2). Chronic treatment with the OX1R antagonist SB-334867, but not with the OX2R antagonist TCS-OX2-29, impaired the acquisition of WIN55,212-2 self-administration, since only 29% of mice receiving SB-334867 reached the acquisition criteria for this behavioural response in comparison with 75% of control animals (Article 2). These acquisition criteria establish the minimum responding values (in terms of stability, amount of drug intake, and preference for the reinforced condition) required to interpret the responding patterns of mice as a consistent operant self-administration and distinguish them from random responding often displayed by mice. Consistent with these pharmacological data, acquisition of operant WIN55,212-2 self-administration was impaired in OX1R knockout mice, since 75% of wildtype animals reached the acquisition criteria in

comparison with 33% of mutant mice. In addition, the responses displayed in the active hole and the subsequent amount of drug intake were reduced in mice lacking the OX1R gene (Article 2). These results suggest that OX1R signalling contributes to the reinforcing properties of WIN55,212-2 required to establish a consistent and stable drug self-administration. In addition, OX1R signalling seems to be involved not only in the acquisition but also in the maintenance of an established operant WIN55,212-2 self-administration. Thus, the acute SB-334867 administration after the acquisition of WIN55,212-2 self-administration blocked this behavioural response, as demonstrated by the decrease in responding on the active hole and the lack of change on inactive nose-poking (Article 2). This result suggests that neuroplastic changes could take place during the learning process of WIN55,212-2 self-administration, leading orexin transmission to be crucial for maintaining operant responding for this synthetic cannabinoid. Moreover, pharmacological blockade of OX1R, as well as its deletion, reduced the maximal effort required to obtain a WIN55,212-2 infusion in a progressive ratio schedule (Article 2), indicating that these neuroplastic changes could also involve cortical structures implicated in motivational processes.

Importantly, SB-334867-treated and OX1R knockout mice displayed a similar operant responding for a natural reward (i.e., water) than the control groups (Article 2). This indicates that the impairment of WIN55,212-2 self-administration observed in OX1R knockout mice or after SB-334867 treatment cannot be attributed to possible unspecific effects of these genetic and pharmacological manipulations, such as locomotor alterations or learning deficits in the acquisition of an operant behaviour. In agreement, it has been previously shown that SB-334867-treated and OX1R knockout mice maintain normal operant responding for standard food reinforcers (Borgland *et al*, 2009; Hollander *et al*, 2012), although they display impaired operant learning when responding for highly palatable rewards (Borgland *et al*, 2009; Cason and Aston-Jones, 2013; Cason *et al*, 2010). However, OX1R manipulations have been reported to alter operant responding for regular food under certain high effort-

demanding schedules of reinforcement (Sharf *et al*, 2010b). Consequently, possible interferences in operant responding for food reinforcement were avoided by using water as a natural reward.

Our behavioural findings point therefore to a specific role for OX1R in the modulation of the reinforcing properties of cannabinoids, since the OX2R antagonist TCS-OX2-29 did not alter this behaviour in any of the self-administration procedures. Both OXR subtypes are localized in brain areas important for reward processing, such as the VTA, NAc, and BNST (Marcus *et al*, 2001), but their role in the modulation of the rewarding properties of drugs of abuse appears to be drug-specific. OX1R and OX2R have been reported to modulate the primary reinforcing effects of opioids (Harris *et al*, 2005; Schmeichel *et al*, 2015; Smith and Aston-Jones, 2012) and alcohol (Brown *et al*, 2013; Lawrence, 2010; Lawrence *et al*, 2006). In contrast, only OX1R contributes to nicotine reinforcement (Hollander *et al*, 2008; Uslaner *et al*, 2014). Notably, primary reinforcing effects of psychostimulants seem to be independent from orexin transmission (Boutrel *et al*, 2005; Smith *et al*, 2009). Indeed, orexins modulate the rewarding properties of cocaine only under conditions that require high degrees of effort and motivation to obtain the drug (España *et al*, 2010; Smith *et al*, 2009). The differential participation of the orexin system in drug-induced reinforcement could be due to the distinct mechanism by which each drug of abuse acts within the reward system. Although all drugs of abuse increase dopamine extracellular levels in the NAc, opioids, nicotine, and alcohol increase dopaminergic cell firing in the VTA, whereas psychostimulants directly inhibit dopamine reuptake in the NAc. Indeed, the VTA shows high density of OX1R (Borgland *et al*, 2009; Narita *et al*, 2006), orexin-A induces a direct depolarization of VTA dopamine neurons (Korotkova *et al*, 2003), and the intra-VTA infusion of orexin-A increases dopamine levels in the NAc (España *et al*, 2011; Narita *et al*, 2006). This suggests that the VTA might be a crucial site of action for orexins to mediate the rewarding effects of different drugs of abuse (Aston-Jones *et al*, 2010). Interestingly, the reinforcing properties of cannabinoids have been associated with their ability to enhance the firing rate and bursting

activity of dopaminergic neurons in the VTA (French *et al*, 1997; Gessa *et al*, 1998; Wu and French, 2000), subsequently enhancing dopamine release in terminal regions, such as the NAc and the PFC (Cheer *et al*, 2004; Chen *et al*, 1990a; Tanda *et al*, 1997). Taking into account these data and our behavioural results, we decided to examine whether the modulation of the reinforcing properties of cannabinoids exerted by orexins relays on VTA dopaminergic transmission. Previous *in vivo* microdialysis studies from our laboratory reported that systemic injection of THC at low doses increased extracellular dopamine levels in the mouse NAc (Robledo *et al*, 2007). Following the same procedure, THC-enhanced dopamine extracellular levels in the NAc of wildtype animals, but this effect was abolished in OX1R knockout mice (Article 2). This suggests that OX1R signalling is required for THC-enhanced dopamine extracellular levels within the NAc, and presumably for cannabinoid-induced reward since it depends to a great extent on increased dopaminergic transmission (Oleson and Cheer, 2012). One possible mechanism explaining this result would be that cannabinoid exposure induces the release of orexins in the VTA following the activation of these neurons in the LH, which directly project to the VTA (Fadel and Deutch, 2002). The subsequent activation of OX1R by orexins in the VTA would be essential for the rewarding properties of cannabinoids through the modulation of the dopamine mesolimbic pathway.

Operant yoked-control models of drug self-administration represent useful tools to distinguish between the neurochemical changes attributable to the pharmacologic actions of the drug and those changes due to operant drug-seeking behaviour (Metaxas *et al*, 2010; Palamarchouk *et al*, 2009). We took advantage of this paradigm to determine whether orexin neurons were in fact activated during WIN55,212-2 self-administration. Contingent self-administration of WIN55,212-2 induced an increase in the percentage of orexin neurons expressing FosB/ΔFosB in the LH when compared with mice receiving saline infusions, indicating repeated activation of this neuronal subpopulation (Article 2). Notably, orexin cells within this region were not

activated in mice receiving passive infusions of the cannabinoid, suggesting that the recruitment of orexin neurons within the LH is mainly due to operant seeking for the reinforcing effects of this drug. The repeated activation of these neurons does not seem to be a consequence of the learning process, since operant conditioning driven by a natural reward did not modify FosB/ΔFosB expression in LH hypocretin cells in comparison with water-yoked mice (Article 2). On the other hand, FosB/ΔFosB expression of orexin neurons within the DMH/PFA region was similar between mice trained to contingently self-administer WIN55,212-2 and those receiving passive infusions of saline. However, a decrease in FosB/ΔFosB expression within DMH/PFA cells was observed in mice receiving WIN55,212-2 passively when compared to those contingently self-administering this synthetic cannabinoid. This effect could be related to a learning process because a higher percentage of activated DMH/PFA orexin cells was found in mice subjected to operant conditioning maintained by water when compared to water-yoked animals (Article 2). Nevertheless, a partial contribution of DMH/PFA orexin neurons in operant seeking behaviour cannot be discarded. It has been proposed that orexin neurons located in these distinct anatomical subregions correspond to two functional populations with different physiological roles, with orexin cells located in the LH being primarily involved in reward processing, and those located in the DMH/PFA mainly regulating arousal and responsiveness to stressful situations (Harris and Aston-Jones, 2006). Moreover, two classes of orexin neurons with distinct electrophysiological features have been described, named “H type” and “D type” according to standard electrical fingerprinting protocols (Schöne and Burdakov, 2012; Schöne *et al*, 2011). In the mouse, the ratio of D to H type orexin neurons is higher in the DMH/PFA compared to the LH (Williams *et al*, 2008). Nevertheless, the projections from LH and DMH/PFA orexin cells, and from H and D types of orexin cells, converge on both the LC and VTA (González *et al*, 2012), suggesting that topographically similar orexin cells could potentially control arousal and reward in the mouse (González *et al*, 2012). Indeed, opposing results have been reported regarding this

possible functional dichotomy of orexin subpopulations. Thus, Fos expression in LH orexin cells was selectively correlated with morphine and cocaine conditioned place preference (Harris *et al*, 2005; Richardson and Aston-Jones, 2012). Similarly, chronic ethanol drinking increased mRNA orexin expression exclusively within the LH (Lawrence *et al*, 2006). However, activation of orexin neurons located in the LH has been also reported during the dysphoric state associated with nicotine (Plaza-Zabala *et al*, 2012a) and morphine (Laorden *et al*, 2012) withdrawal. Our results support a partial functional dichotomy of orexin neurons based on their anatomical location, since WIN55,212-2 reinforcing effects were associated with a selective activation of LH orexin neurons in the present study. Still, high basal activation of these cells was observed in contingent/non-contingent operant conditioning maintained by water (both in LH and DMH/PFA) probably due to the stress state induced by water deprivation (Article 2; Csaba *et al*, 2005; Ruginsk *et al*, 2015). Thus, anatomically distinct orexinergic populations might differ functionally only when regulating certain physiological processes.

The possible consequences of ΔFosB expression in orexin neurons during WIN55,212-2 self-administration remain rather unknown. ΔFosB is upregulated in numerous brain regions following repeated drug exposure, including cannabinoids (Lazenka *et al*, 2014), and its induction has been suggested to contribute to the mechanisms underlying addiction (Nestler, 2001; Ruffle, 2014). Among the numerous target genes of this transcription factor, of special interest is the opioid peptide dynorphin, which is suppressed by ΔFosB (Zachariou *et al*, 2006). Dynorphins have been associated with drug-induced aversion, including that produced by cannabinoids (Mendizábal *et al*, 2006). A large proportion of orexin neurons potentially co-release orexin and dynorphin peptides (Chou *et al*, 2001), and it has been suggested that orexins facilitate reward by attenuating the antireward effects of its co-transmitter dynorphin in the VTA (Muschamp *et al*, 2014). Thus, inhibition of dynorphin expression in orexinergic neurons would facilitate orexin transmission to contribute to the rewarding properties of cannabinoids, supporting the maintenance of

cannabinoid-seeking behaviour. This mechanism could partly underlie the neuroadaptations in orexin transmission that contribute to the maintenance of WIN55,212-2 self-administration (Article 2).

Our results showing cannabinoid-induced activation of orexin neurons are in divergence with previous electrophysiological data reporting that bath application of the synthetic cannabinoid WIN55,212-2 indirectly inhibits the firing rate of orexin cells by CB1R-mediated presynaptic attenuation of glutamate release (Huang *et al*, 2007). Indeed, CB1R is predominantly located in excitatory inputs to orexin neurons (Cristino *et al*, 2013). This particular location of CB1R would explain cannabinoid-induced inhibition of orexin firing (Huang *et al*, 2007), as well as the trend to decrease FosB/ΔFosB expression after chronic passive exposure to WIN55,212-2 in orexins within the DMH/PFA (Article 2). However, a recent study supports that this CB1R-mediated control of GABAergic and glutamatergic inputs to orexin neurons can be switched under certain conditions (Cristino *et al*, 2013). Thus, orexin neurons appear to receive predominantly inhibitory instead of excitatory CB1R-expressing inputs in leptin knockout obese mice and in diet-induced obese mice, promoting disinhibition of orexin transmission by local endocannabinoid release (Cristino *et al*, 2013), and likewise by exogenous cannabinoid exposure. It is therefore reasonable that if this synaptic remodelling takes place in certain pathological conditions, such as obesity, the development of drug addiction might entail similar consequences through different mechanisms. Thus, analogous neuroadaptive changes could take place during the acquisition of WIN55,212-2 self-administration to promote the activation of orexin neurons, contributing to maintain cannabinoid-seeking behaviour. This maintenance could involve orexin transmission within the VTA and subsequent dopamine release, since OX1R signalling seems to be involved in cannabinoid-induced stimulation of dopaminergic transmission (Article 2). However, other structures in the mesocorticolimbic system, such as the PFC, might also mediate the maintenance of cannabinoid-seeking, since orexins have been reported to increase glutamatergic prefrontal control of dopamine release (Moorman and Aston-Jones, 2010).

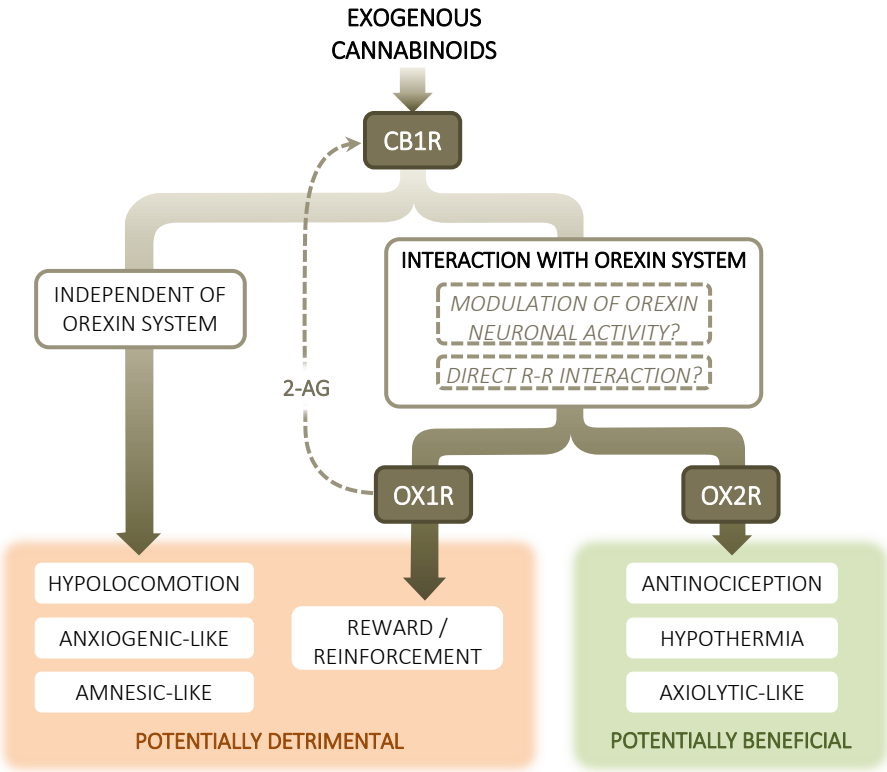

Figure 16. Orexinergic modulation of cannabinoid-induced behavioural effects. Schematic diagram summarizing our main findings regarding orexinergic modulation of acute pharmacological and reinforcing effects induced by exogenous cannabinoid exposure. Exogenous cannabinoids, such as THC or WIN55,212-2, produce a series of behavioural responses, presumably through CB1R. Some of these responses are independent of orexin transmission, such as hypolocomotion, anxiogenic-like and amnesic-like effects. However, antinociception, hypothermia and anxiolytic-like responses are modulated by OX2R signalling. Moreover, OX1R stimulation contributes to cannabinoid-induced reinforcement. Thus, the potentially beneficial effects of cannabinoid-based therapies could be preserved if OX1R blockade was employed as a therapeutic tool to avoid the addictive properties of cannabinoids. Possible mechanisms involved in orexinergic modulation of cannabinoid-induced responses (discontinous lines) include the activation of orexin release by cannabinoids, the direct molecular interaction between cannabinoid and orexin receptors and subsequent alteration in downstream signalling, or the potentiation of CB1R signalling by OX1R-induced 2-AG biosynthesis. 2-AG, 2-arachidonoylglycerol.

In summary, our data reveal a differential role for each orexin receptor subtype in several effects induced by cannabinoids (Figure 16). Thus, OX2R signalling contributes to THC-induced hypothermia, supraspinal antinociception and anxiolytic-like effects, whereas the reinforcing properties of the synthetic cannabinoid WIN55,212-2 are controlled by

OX1R signalling. In addition, OX1R appears to be involved in the ability of THC to enhance dopamine extracellular levels in the NAc, a mechanism that could contribute to cannabinoid reinforcement. Moreover, operant WIN55,212-2 self-administration promotes the activation of orexin neurons in the LH, potentially participating in the maintenance of cannabinoid-seeking behaviour.

2. Participation of the orexin system in the consolidation and extinction of fear memories

Coping with dangerous or stressful situations involves the emergence of transient and physiological fear and anxiety responses, as well as the formation of aversive memories that allow the individual to confront or avoid potential threats in the future. Therefore, emotional responsiveness and emotional memory processing are an adaptive phenomenon necessary for individuals to survive. However, if fear or anxiety responses to threatening stimuli persist beyond the adaptive level, typically due to excessive aversive conditioning or resistance to fear extinction, these emotional states can become pathological, leading to anxiety disorders, such as PTSD and phobias (Holmes and Singewald, 2013; Kindt, 2014). Substantial evidence has demonstrated the role of orexins as mobilizers of physiological responses to emotional and stressful stimuli, including cardiovascular stimulation and increased arousal (Article 5). However, few reports have examined the possible role of orexins in fear memory processing. Previous studies have shown that fear conditioning increases prepro-orexin mRNA levels in rats (Chen *et al*, 2014), and the DORA almorexant reduced fear-potentiated startle responses when administered before retrieval (Steiner *et al*, 2012). Although these findings point to an orexinergic modulation of fear memory, they might reflect a modulation of fear expression instead. In this thesis, we demonstrate that orexins have a crucial role in fear memory processing, promoting the consolidation of fear memories, as well as the resistance to fear extinction through OX1R signalling.

We used the fear conditioning paradigm and employed either contextual or auditory cues as conditioning stimuli. In both paradigms, pharmacological blockade of OX1R with low doses of SB-334867 immediately after the conditioning trial strongly decreased freezing behaviour during the test session 24h later (Article 4). Similar results were obtained in OX1R knockout mice, confirming the role of this receptor in the formation of contextual and cued fear memories (Article 4). Consistent with previous reports, OX1R knockout mice showed similar pain perception, locomotor activity, and anxiety-like behaviour than wildtype animals (Article 1; Scott *et al*, 2011; Soya *et al*, 2013), suggesting that the differences between the two genotypes in fear memory formation were not due to alterations in sensory-motor abilities of mice lacking the OX1R gene. In contrast, the administration of the OX2R antagonist TCS-OX2-29 only impaired contextual fear conditioning (Article 4). Importantly, OX1R and OX2R antagonists did not modify freezing behaviour when administered 4h after the fear conditioning trial (Article 4). This result demonstrates that: (1) orexin signalling is necessary during the early consolidation phase of aversive memories, and (2) the low freezing levels displayed during the test session are not due to the influence of orexin antagonists in the expression of fear/anxiety responses, but to their influence in fear memory processing. Indeed, SB-334867 or TCS-OX2-29 acute treatments at the doses used for the fear conditioning experiments had no effect in locomotion or anxiety-like behaviour when administered 24h before testing (Article 4). These data also show a different role for OX1R and OX2R in the consolidation of cued and contextual fear memories, in agreement with recent data using knockout mice (Soya *et al*, 2013). This divergent response could be explained by the different distribution of these receptors throughout the brain. Thus, OX1R is substantially expressed in the hippocampus, a crucial region for the consolidation of contextual fear memories (Maren *et al*, 2013), and also in the amygdala, which is involved in both contextual and cued fear conditioning (Flavell and Lee, 2012; Johnson *et al*, 2012a; Maren *et al*, 2013). In contrast, OX2R expression in the hippocampus is high, but

relatively low in the amygdala (Johnson *et al*, 2012a). Recent studies in rats showed also a specific role for OX1R in the formation of auditory threat-conditioned memories (Sears *et al*, 2013), as revealed by the reduction in freezing levels when SB-334867, but not TCS-OX2-29, was centrally infused before the fear conditioning trial. However, this study reports that OX1R signalling is required for the acquisition phase of threat learning, but not for the consolidation phase, since OX1R antagonism after the conditioning session had no significant effects on freezing levels (Sears *et al*, 2013). Despite this divergence with our findings, probably due to different experimental conditions and animal species, it seems clear that orexin transmission is crucial for the intact formation of threat memories. Notably, we did not observe any modification in the consolidation of object-recognition memory due to OX1R deletion or pharmacological blockade (Article 4), according to previous observations (Article 1) supporting the idea that orexins are primarily involved in the consolidation of highly emotional memories.

Taking into account that the stressful events that trigger the development of some anxiety disorders, such as PTSD and phobias, are in general unpredictable, pharmacological interventions should be preferentially directed towards facilitation of fear memory extinction, rather than excessive memory formation. For this reason, we also evaluated the possible involvement of orexins in the extinction of fear memories. OX1R and OX2R antagonists, as well as orexin-A and orexin-B peptides, were administered once the aversive memory had already been successfully formed, after each session of the extinction period. We found that OX1R blockade with SB-334867 strongly facilitated fear extinction in both cued and contextual fear conditioning paradigms, whereas orexin-A infusion promoted resistance to extinguish conditioned fear (Article 4). On the contrary, OX2R blockade and orexin-B infusion were ineffective, suggesting that orexin-A has a key role in the extinction of aversive memories probably through specific OX1R activation. Notably, administration of orexin-A in naïve mice did not alter locomotor activity or anxiety-like responses when evaluated the following day, excluding that

scored freezing levels were affected by possible anxiety-promoting effects of orexin-A infusion (**Article 4**). Pharmacologic or genetic manipulations that modify learning and memory generally affect both memory formation and extinction processes in a similar manner. Thus, glucocorticoids increase fear memory formation and facilitate fear extinction (Rodrigues *et al*, 2009), and similar results are obtained upon TrkB stimulation (Andero *et al*, 2011). In contrast, orexin transmission seems to differently affect the consolidation of fear memory formation and extinction, since orexin signalling is required for appropriate fear memory formation, whereas it hinders posterior extinction of these fear memories. However, molecular signalling through other neurochemical systems, such as TrkC (Santos *et al*, 2015) or PKA (Nijholt *et al*, 2008), has also been reported to present this particular pattern of fear memory modulation. Interestingly, we observed an increased c-Fos expression in orexin neurons within both the LH and the DMH/PFA during the contextual fear extinction process in C57BL/6J animals, indicating that these neurons were being activated during the extinction sessions. Together with the behavioural data, these biochemical results could indicate that orexin neurons are engaged to preserve fear, counteracting the normal physiological process of fear extinction. Chronic OX1R blockade could also be important to maintain the facilitation of fear memory extinction given that orexin neurons remain activated during the whole extinction process. The role of the orexin system in fear extinction was further confirmed in the 129S1/SvImJ mice, a strain that exhibits a profound impairment of fear extinction (Camp *et al*, 2012; Hefner *et al*, 2008) and is considered a valuable model for identifying extinction-facilitating drugs (Gunduz-Cinar *et al*, 2013; Whittle *et al*, 2010). OX1R blockade rescued deficient extinction of both cued and contextual fear memories in 129S1/SvImJ mice. This finding suggests that impairment of fear extinction exhibited by this mouse strain could be related to a hyperfunction of the orexin system, similarly to what has been observed in certain anxiety disorders (Johnson *et al*, 2010), although this possibility remains to be confirmed. Taken together, our findings support the

potential usefulness of OX1R as a pharmacological target in diseases characterized by inappropriate maintenance of aversive memories.

Among the numerous brain regions involved in emotional memory processing, the infralimbic PFC, the hippocampus and the BLA constitute key structures in the neurobiological circuit underlying fear extinction (Herry *et al*, 2008; Milad and Quirk, 2012; Article 5). Thus, the infralimbic PFC and the hippocampus activate the inhibitory circuits of the amygdala during extinction learning to reduce fear expression (Herry *et al*, 2008). Diverse studies have examined the activation patterns of these brain regions during fear extinction by quantifying immediately-early gene (c-Fos and Zif268) expression. These studies conclude that impaired fear extinction is associated with reduced immediate-early gene expression in the corticoamygdalar circuit (Herry and Mons, 2004; Herry *et al*, 2010; Holmes and Singewald, 2013), whereas a complete extinction of conditioned fear is related to increased immediate-early gene levels in these brain areas (Herry and Mons, 2004). In agreement, a number of animal models presenting resistance to fear extinction, such as the 129S1/SvImJ mouse strain (Hefner *et al*, 2008), mice lacking dynorphin (Bilkei-Gorzo *et al*, 2012), or a poor-extinction subpopulation of C57BL/6J mice (Herry and Mons, 2004), showed reduced activation of the infralimbic PFC and the BLA. Consistently, human studies employing fMRI revealed that hypoactivity in the ventromedial PFC (the functional analogous to infralimbic PFC) may contribute to the impairment of extinction observed in PTSD and phobias (Hermann *et al*, 2009; Milad *et al*, 2009; Shin *et al*, 2005). We found that the contextual extinction-facilitating effects of SB-334867 were associated with increased c-Fos expression in both the infralimbic PFC and the BLA (Article 4), suggesting that the blockade of OX1R signalling might increase the inhibitory control from the infralimbic cortex over the BLA during this process, facilitating the activation of the amygdalar inhibitory circuits involved in fear extinction. Notably, the association between facilitation of impaired fear extinction and increased infralimbic activity has been observed also in human studies, reporting that cognitive-behavioural therapy increases

ventromedial PFC blood flow in panic disorder (Sakai *et al*, 2006). We also found that SB-334867 administration did not modify c-Fos expression in the prelimbic PFC and the dorsal hippocampus during contextual fear extinction (Article 4). Accordingly, the prelimbic PFC appears to have an opposite role to the infralimbic portion, being implicated in sustained fear expression and resistance to extinction of aversive memories (Sierra-Mercado *et al*, 2011), and has been reported to present either decreased or unaltered activation during fear extinction (Bilkei-Gorzo *et al*, 2012; Whittle *et al*, 2010). On the other hand, several studies report that the dorsal hippocampus is crucial for contextual fear extinction (Ji and Maren, 2005, 2007; Nijholt *et al*, 2008; Stafford *et al*, 2012). However, orexin transmission does not modulate the activation state of this brain region during fear extinction, although other possible orexigenic actions within this structure could not be discarded.

This SB-334867-induced activation pattern throughout the fear circuit could denote the existence of direct OX1R stimulation during contextual fear extinction in certain brain regions, particularly the BLA and the infralimbic cortex. As previously mentioned, orexin fibres (Peyron *et al*, 1998) and OX1R (Marcus *et al*, 2001) are moderately distributed within the BLA (Supplementary Results), both prelimbic and infralimbic PFC, and hippocampus, indicating that orexins might potentially act through any of these structures. Interestingly, SB-334867 infusion in the BLA facilitated extinction of contextual fear memory, indicating that OX1R located within this brain region are directly involved in this process (Article 4). Nevertheless, this effect was not as prominent as the one observed with systemic administration, suggesting that other brain regions might contribute to the extinction-facilitating effects of the OX1R antagonist. The infusion of SB-334867 did not produce any effect when administered in the infralimbic cortex (Article 4), suggesting that the increased activity of this nucleus associated with the extinction-facilitating actions of OX1R blockade was an indirect consequence. The PVT could be one possible candidate to modulate this effect, since this brain area has numerous projections to the infralimbic PFC and at the same time contains high

density of OX1R (Marcus *et al*, 2001), which stimulation induces fear and anxiety-like behaviour in rodents (Hsu *et al*, 2014; Li *et al*, 2010). However, recent studies show that OX1R blockade in the PVT has no effect on the expression of conditioned fear in rats (Dong *et al*, 2015), and lesioning this structure appears not to affect fear extinction (Li *et al*, 2014). These results suggest that other structures might be mediating the potential indirect effect of orexins in the infralimbic PFC. On the other hand, direct OX1R blockade within the dorsal hippocampus confirmed that OX1R signalling within this brain region is not involved in the modulation of contextual fear extinction (**Article 4**). The dorsal hippocampus has been largely associated with contextual fear extinction and local injection of diverse compounds within this structure has been reported to facilitate fear extinction (Nijholt *et al*, 2008; Stafford *et al*, 2012; Tronson *et al*, 2008). However, the ventral subdivision of the hippocampus also appears to be crucial for this process (Sierra-Mercado *et al*, 2011), has more projections to the PFC and the BLA than the dorsal portion (Hoover and Vertes, 2007; Pitkänen *et al*, 2000), and also presents high expression of OX1R (Hervieu *et al*, 2001). Therefore, the possible participation of the ventral hippocampus in the orexinergic modulation of fear extinction should be further addressed in the future.

Two distinct populations of BLA neurons encode fear conditioning and extinction processes (Herry *et al*, 2008). These subpopulations, functionally identified as ‘‘extinction neurons’’ and ‘‘fear neurons’’, overlap with two distinct anatomically defined subpopulations of BLA neurons projecting to infralimbic or prelimbic cortices, respectively (Senn *et al*, 2014). One possible mechanism by which orexins could interfere with fear extinction is the modulation of amygdalar activity and its connection with the infralimbic cortex. Therefore, SB-334867-induced enhancement in BLA c-Fos expression during contextual fear extinction (**Article 4**, **Supplementary Results**) could reflect an increase of the recruitment of extinction-related neurons, which in turn would increase the activation state of the infralimbic PFC. Differential identification of prelimbic- and infralimbic-projecting neurons through retrograde labelling

revealed that SB-334867 treatment modified the activation pattern of PFC-projecting BLA neurons observed in vehicle-treated animals during contextual fear extinction (Supplementary Results). Thus, whereas control animals showed a higher activation of prelimbic- than infralimbic-projecting neurons, mice treated with the OX1R antagonist presented a trend in the opposite direction. Indeed, SB-334867-treated mice displayed a higher number of c-Fos+ infralimbic-projecting neurons than the vehicle group. Interestingly, the OX1R antagonist might be enhancing the activation of other neurons different to those projecting to the PFC regions (e.g., local interneurons), since the number of non-retrolabelled c-Fos+ neurons was also higher in the SB-334867 group. Moreover, orexin projections were detected in close proximity to both prelimbic- and infralimbic-projecting neurons, suggesting that orexin neurons might potentially establish direct connections with these cells (Supplementary Results). These results, together with previous data showing fear extinction acceleration by intra-amygdalar infusion of SB-334867 (Article 4), suggest that orexins might directly act in the amygdala, hindering the activation of BLA neurons that project to the infralimbic cortex and impairing fear extinction.

The molecular mechanisms by which orexin transmission modulates fear extinction remain rather unclear. Some of the main molecular pathways triggered upon orexin receptor stimulation have also been associated with regulation of fear memory. Thus, a number of reports suggest that PKC signalling is associated with increased fear conditioning. Overexpression of PKMζ, a specific PKC isoform, in the prelimbic PFC enhanced the formation of auditory fear (Xue *et al*, 2015), whereas PKCβ knockout animals exhibited deficits in both cued and contextual fear conditioning (Weeber *et al*, 2000). In addition, intra-BLA infusion of H7, a potent inhibitor of both PKC and protein kinase A, reduced freezing levels in these paradigms (Goosens *et al*, 2000). Interestingly, PKC might also be involved in contextual fear extinction, since the PKC inhibitor NPC-15437 accelerated this behavioural response when administered by systemic (Supplementary Results) or intra-hippocampal route (Tronson *et*

al, 2008). These results suggest that PKC signalling delays the fear extinction process and might potentially contribute to resistance to extinction induced by orexin-A. Indeed, pre-treatment with a subeffective dose of NPC-15437 successfully prevented the extinction-resistance induced by orexin-A exposure (**Supplementary Results**), suggesting that PKC activation upon orexin-A infusion could contribute to slow down the extinction process.

On the other hand, release of the endocannabinoid 2-AG represents another potential mediator of orexin-induced fear extinction impairment. Although endocannabinoids have been associated with facilitation of fear extinction, recent reports demonstrate that endocannabinoid signalling might enhance the expression of fear under certain circumstances. Indeed, increasing 2-AG levels by systemic administration of the MAGL inhibitor JZL184 before retrieval of auditory conditioned fear enhanced freezing behaviour (Hartley *et al*, 2016; Llorente-Berzal *et al*, 2015), whereas increased anandamide levels by systemic injection of the FAAH inhibitor URB597 had the opposite effect (Llorente-Berzal *et al*, 2015). Interestingly, when injected right after retrieval sessions, JZL184 impaired contextual fear extinction, although URB597 had no effect (**Supplementary Results**). These data suggest that, whereas high anandamide concentration reduces fear expression, increased 2-AG levels promote fear expression and also impair consolidation of fear extinction. It seems that fear expression-promoting actions of 2-AG might take place through CB1R signalling, since the CB1R antagonist rimonabant blocked these effects of JZL184 (Hartley *et al*, 2016; Llorente-Berzal *et al*, 2015). This result was also observed in mice lacking CB1R specifically in GABAergic neurons, suggesting that this neuronal population is responsible for 2-AG-induced fear expression (Llorente-Berzal *et al*, 2015). Deletion of CB1R receptor from habenular neurons also reduces fear-conditioned freezing (Soria-Gómez *et al*, 2015), indicating that CB1R signalling might promote fear expression when acting at diverse neuronal subtypes. Since orexin-A infusion produced similar effects to those produced by increased 2-AG levels, this endocannabinoid could be contributing to orexinergic

resistance to fear extinction. Indeed, blockade of 2-AG biosynthesis by central infusion of the DAGL inhibitor O-7460 reduced the effects of orexin-A and prevented extinction resistance (Supplementary Results). However, this effect was maintained only during the initial phases of the experimental procedure. In concordance, it has been reported that 2-AG augmentation impaired extinction behaviour primarily during a short temporal window (Hartley *et al*, 2016), suggesting a preferential contribution of 2-AG to short-term phases of extinction resistance induced by orexin-A. The effects of JZL184 in fear expression appear to be partly due to augmentation of 2-AG signalling in the BLA, as direct microinfusion of JZL184 into this structure produced similar results (Hartley *et al*, 2016). Interestingly, central infusion of orexin-A at the same dose that induces impairment of fear extinction increased 2-AG levels in the amygdala of naïve mice (Supplementary Results). This enhanced 2-AG presence in the amygdala took place 10 minutes after orexin infusion, which could correspond to direct orexin receptor stimulation with subsequent 2-AG release within this brain region. In contrast, analysis of PFC samples revealed that cortical 2-AG increased at 30 minutes after orexin-A infusion, a fact that might be interpreted as an indirect effect of orexin, rather than direct orexin-mediated stimulation of 2-AG biosynthesis (Supplementary Results). Notably, an inverse correlation was observed between 2-AG levels detected in the amygdala and the PFC at 30 minutes after orexin infusion (Supplementary Results). Together with previous behavioural and biochemical findings, these results suggest that orexin-A might directly act within the BLA to indirectly modulate the PFC by altering the communication between these two brain structures. In accordance, recent human studies report that individuals with clinically relevant anxiety levels showed a negative coupling between amygdala resting state activity and ventromedial PFC activity (Kim and Whalen, 2009; Kim *et al*, 2011), suggesting that orexin signalling may contribute to the dysfunctions observed in connectivity between these two brain regions that increase susceptibility to anxiety disorders.

In summary, our results reveal that both OX1R and OX2R are involved in the consolidation of fear memories, but only OX1R participates in fear extinction processes. Orexins appear to delay fear extinction through direct OX1R signalling within the BLA, hindering the activation of extinction neurons, among other cells, that recruit the infralimbic PFC (Figure 17). Moreover, orexins might potentially exert this effect through 2-AG release in the BLA. In addition, the activation of PKC pathway is also involved in orexin-induced resistance to fear extinction.

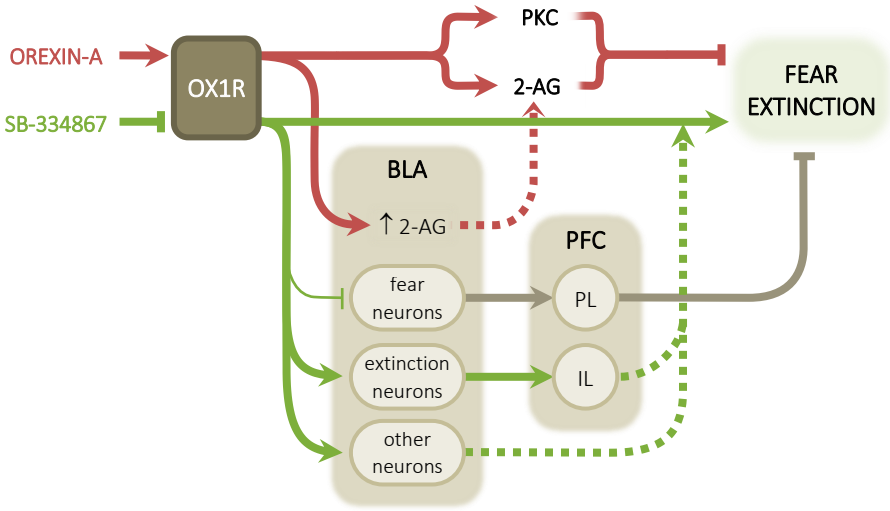

Figure 17. Role of the orexin system in fear extinction. These schematic diagram summarizes our main findings regarding orexinergic modulation of contextual fear extinction. Blockade of OX1R with the selective antagonist SB-334867 (green arrows) promotes fear extinction, partly by direct action within the BLA. SB-334867 enhances the activation of extinction IL-projecting neurons (among others) in the BLA, recruiting also the IL cortex, a mechanism that could contribute to its extinction-facilitating effects. OX1R blockade tends also to decrease fear PL-projecting BLA neurons, although SB-334867 does not seem to modify PL activity (grey arrows). Orexin-A (red arrows), likely through OX1R stimulation, leads to fear extinction impairment. OX1R-induced PKC pathway and 2-AG biosynthesis contribute to this effect. Moreover, orexin-A induces a transient increase in 2-AG levels in the BLA, which could favour impairment of fear extinction. Continous lines, experimental results or available evidence; discontinous lines, speculative relations. 2-AG, 2-arachidonoylglycerol; BLA, basolateral amygdala; IL, infralimbic prefrontal cortex; PL, prelimbic prefrontal cortex; PKC, protein kinase C.

3. Concluding remarks

The current thesis has identified new neuromodulatory roles for the orexin system that support their fundamental involvement in motivational and emotional processes, such as drug-seeking behaviour or aversive memory. Indeed, a common substrate underlying these two apparently independent processes is recruitment of the orexin system, triggered either by external signals of reward or threat. In addition, our results suggest that in both situations the orexin system might collaborate at some particular points with the endogenous cannabinoid system (**Articles 1** and **2**, **Supplementary Results**), supporting the relevance of the interaction between these two systems (**Article 3**) and encouraging further research to elucidate these mechanisms. We have observed a differential modulation of cannabinoid-induced effects by orexin receptor subtypes that might entail relevant therapeutic implications. Indeed, the rewarding effects of cannabinoids are crucial in the initiation of their use and dependence. Taking into account the involvement of OX1R in cannabinoid reinforcement (**Article 2**), OX1R antagonists arise as a new possible therapeutical target that could prevent progression from sporadic cannabis use to chronic misuse. The specific involvement of OX2R, but not of OX1R, in the modulation of other acute pharmacological responses induced by THC (**Article 1**) reveal the possibility of OX1R blockade to abolish the reinforcing properties of cannabinoids without affecting other pharmacological responses of these compounds interesting at the therapeutical level. This might be of special interest in those conditions in which medical use of cannabinoid-like compounds is indicated, since co-therapy with OX1R antagonists might prevent the emergence of cannabinoid addiction in these situations. On the other hand, the enhancement of aversive memory extinction shown by OX1R blockade (**Article 4**), together with its previously reported effects in anxiety and fear expression, suggest that OX1R antagonists could also be a promising therapeutical approach for the treatment of some anxiety disorders, such as PTSD and phobias (**Article 5**). The prevalence of comorbid substance use and anxiety disorders is relatively high, and co-

occurrence of both psychiatric disorders has a large clinical impact in the course and treatment outcome of the counterpart condition (Smith and Book, 2008). Therefore, OX1R blockade could be particularly useful in these cases, since it might also prevent the development of cannabis addiction in subjects with anxiety disorders that could misuse cannabis as a self-medication strategy.

CONCLUSIONS

The findings revealed in the present thesis allow to draw the following conclusions:

1. Orexin peptides modulate THC-induced hypothermia, supraspinal antinociception and anxiolytic-like effects, likely through OX2R stimulation. Other behavioural effects induced by acute THC administration, such as hypolocomotion, spinal antinociception, anxiogenic- and amnesic-like effects are independent of orexin signalling.
2. Prepro-orexin knockout mice present reduced activation of the central amygdala, preoptic area and lateral septum upon THC administration, suggesting that orexin transmission within these brain regions might contribute to THC-induced acute pharmacological effects.
3. Orexin peptides, acting specifically through OX1R, modulate the reinforcing properties of the synthetic cannabinoid WIN55,212-2, controlling the acquisition and maintenance of WIN55,212-2 self-administration behaviour, as well as the motivation to obtain the drug. In contrast, OX1R does not influence operant conditioning maintained by a natural reward, suggesting that OX1R plays a specific role in cannabinoid-induced reinforcement.
4. OX1R is involved in the ability of THC to enhance dopamine levels within the NAc, a mechanism that contributes to the modulation of cannabinoid rewarding effects.
5. Operant WIN55,212-2 self-administration is associated with activation of orexin cells in the LH, suggesting that these neuronal population is recruited during cannabinoid seeking. The activation of these neurons might contribute to this behavioural response by enhancing cannabinoid-induced dopaminergic transmission. Notably, orexin neurons within the LH are not activated as a consequence of cannabinoid exposure *per se* or the learning process involved in operant conditioning.
6. Orexin transmission contributes to the consolidation of contextual fear memory through both OX1R and OX2R signalling, whereas only

OX1R is involved in the consolidation of cued fear memory. Moreover, orexins preferentially modulate the formation of memories with high emotional relevance.

7. Orexin peptides through OX1R signalling delay fear extinction elicited by exposure to both contextual and cued fear-associated stimuli. In agreement, orexin neurons are recruited during contextual fear extinction, presumably to counteract this physiological process. Moreover, OX1R blockade facilitates fear extinction and reduces fear expression in a mouse model of impaired extinction.
8. Systemic OX1R blockade enhances the activation of the BLA and the infralimbic PFC associated to contextual fear extinction. In addition, the extinction-facilitating effects of OX1R antagonism are exerted, at least in part, through direct OX1R signalling within the BLA, independently from infralimbic and hippocampal OX1R. Enhancing the activation of extinction neurons within the BLA that recruit the infralimbic PFC might be a mechanism contributing to the extinction-promoting effects of OX1R blockade.
9. Orexin-mediated activation of the PKC pathway and 2-AG release contribute to the impairment of fear extinction induced by orexins. Moreover, orexins might potentially affect amygdalocortical communication through 2-AG release in the BLA to promote fear extinction resistance.
10. OX1R signalling significantly contributes to motivational and emotional behaviours involved in the pathophysiology of certain psychiatric conditions, such as drug addiction and anxiety disorders. The specific role of OX1R in cannabinoid-induced reinforcement highlights the potential of OX1R antagonists as new therapeutical tools to prevent the development of cannabis addiction. On the other hand, the involvement of OX1R in aversive memory processing underlines the potential value of OX1R antagonists as extinction-enhancing agents for the treatment of anxiety disorders characterized by abnormal fear persistence, such as PTSD and phobias.

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ANNEX

ARTICLE 6

Hypocretin/orexin signalling in the hypothalamic paraventricular nucleus is essential for the expression of nicotine withdrawal

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ARTICLE 7

A role for hypocretin/orexin receptor-1 in cue-induced reinstatement of nicotine-seeking behavior

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ARTICLE 8

Influence of δ-opioid receptors in the behavioural effects of nicotine

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ARTICLE 9

Role of β4 nicotinic acetylcholine receptors in the habenulo-interpeduncular pathway in nicotine reinforcement in mice

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ARTICLE 10

Hypocretins/orexins and addiction: role in cannabinoid dependence

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Abstract

Hypocretins/orexins are hypothalamic neuropeptides which regulate physiological processes such as energy balance, stress and arousal, sleep/wake cycle, and emotional states. Moreover, a great body of evidence indicates that the hypocretinergic system modulates the addictive properties of several drugs of abuse. Cannabis is the most widely used illicit drug in the word. However, no accepted pharmacologic treatment is available to facilitate and maintain abstinence. Here we describe that the hypocretin receptor 1 is necessary for the rewarding properties of the synthetic cannabinoid WIN55,212-2. Chronic exposure to this synthetic cannabinoid affects the activity of hypocretin neurons in the lateral hypothalamus. Moreover, the enhancement in dopamine extracellular levels in the nucleus accumbens induced by Δ^9-tetrahydrocannabinol, which is related to the rewarding properties of this drug, is blocked in mice lacking the hypocretin receptor 1. Therefore, hypocretin receptor antagonists could represent an interesting pharmacological tool for the treatment of cannabis dependence in humans.

Key Words: hypocretin/orexin, cannabinoid, lateral hypothalamus, reward, self-administration, mice, dopamine

List of abbreviations

2-AG 2-arachidonoylglycerol

cAMP cyclic adenosine monophosphate

CB1 cannabinoid receptor 1

CB2 cannabinoid receptor 2

CNS central nervous system

DAG diacylglycerol

DMH dorsomedial hypothalamus

GABA γ-aminobutyric acid

HcrtR1 hypocretin receptor 1

HcrtR2 hypocretin receptor 2

HcrtRs hypocretin receptors

IP3 inositol trisphosphate

LH lateral hypothalamus

MAP mitogen-activated protein

mRNA messenger ribonucleic acid

NAc nucleus accumbens

PFA perifornical area

THC Δ^9-tetrahydrocannabinol

VTA ventral tegmental area

The hypocretin/orexin system

Hypocretin-1/orexin-A and hypocretin-2/orexin-B are two neuropeptides proteotically cleaved from the same precursor, prepro-hypocretin/prepro-orexin (de Lecea et al 1998; Sakurai et al 1998). Two closely related G protein-coupled receptors that respond to hypocretins have been cloned, hypocretin or orexin receptor 1 (HcrtR1/OXR1) and hypocretin or orexin receptor 2 (HcrtR2/OXR2) (Figure 1). Hypocretin-1 binds to both receptors with similar affinity, whereas hypocretin-2 binds to HcrtR2 with 10-fold greater affinity than to HcrtR1. The intracellular signaling pathways that mediate the effects of hypocretins have been intensively investigated but remain still to be completely elucidated. Stimulation of both hypocretin receptors (HcrtRs) leads to a prominent increase in intracellular Ca^{2+} concentrations through the activation of Gq proteins followed by phospholipase and subsequent protein kinase C (PKC) stimulation (Xu et al 2013). The Ca^{2+} elevation induced by hypocretin receptor activation explains the commonly reported neuroexcitatory nature of hypocretin peptides on the brain. In addition, hypocretins have been reported to activate the MAP kinase pathway (Xu et al 2013) (Figure 2).

In the central nervous system (CNS), hypocretin expression is restricted to a few thousand neurons in some particular regions of the hypothalamus: the perifornical area (PFA), the dorsomedial hypothalamus (DMH), and the dorsal and lateral hypothalamus (LH) (Peyron et al 1998). Although hypocretin-containing neurons represent a relatively small number of cells, their projections are widely distributed throughout the brain (Peyron et al 1998) (Figure 1). The widespread extension of the hypocretin system in the CNS is in agreement with the variety of physiological functions of hypocretin peptides, that includes energy homeostasis, behavioural arousal, sleep/wake cycles and emotional regulation (Li et al 2014). Moreover, numerous studies demonstrate a role for hypocretins in reward-seeking and addiction (Plaza-Zabala et al 2012). Consistent with this, hypocretin neurons send significant efferent projections to structures related to addiction such as the paraventricular nucleus of the thalamus,

the septal nuclei, the bed nucleus of the stria terminalis, the anterior and central amygdaloid nuclei, and the ventral tegmental area (VTA). Disperse axons are also found throughout the cortex and the medial part of the nucleus accumbens (NAc) (Peyron et al 1998). Moreover, hypocretin-1 injected into the VTA increases dopamine in the NAc (España et al 2010), suggesting that these neuropeptides play a role in the regulation of the dopaminergic mesocorticolimbic system and reward learning.

Role of the hypocretin/orexin system in the addictive properties of drugs of abuse

As mentioned above, the hypocretin system plays a crucial role in the regulation of the addictive properties of several major drugs of abuse (Figure 3). Hypocretin transmission regulates the primary reinforcing effects of opioids, nicotine and alcohol (Plaza-Zabala et al 2012). Recent evidence also suggests a role for these neuropeptides in cannabinoid reward (Flores et al 2014). However, in the case of psychostimulants, an involvement of the hypocretin system is revealed only when effort requirement to obtain the drug is high (España et al 2010). The differential participation of hypocretins in the reinforcing properties of psychostimulants could be explained by the different action upon the mesolimbic system. Thus, opioids, nicotine, alcohol, and cannabinoids increase dopaminergic cell firing in the VTA whereas psychostimulants directly inhibit dopamine uptake in the NAc. These differences suggest that the VTA might be a crucial site of action for hypocretins to mediate the rewarding effects of different drugs of abuse (Aston-Jones et al 2009). Therefore, behavioural effects that depend on increased VTA dopaminergic activation (opioid/alcohol/nicotine/cannabinoid reinforcement) would be attenuated by HcrtR1 antagonist because they may required hypocretin transmission. However, behaviours which are independent of the activation of VTA (primary cocaine reinforcement) could avoid this critical site of actions of hypocretins. Moreover, hypocretins regulate the reinstatement of drug-seeking behaviours induced by drug-associated cues as described for cocaine, alcohol, heroin and nicotine. Stress-induced

reinstatement of cocaine and alcohol is also mediated by hypocretin transmission (Khoo and Brown, 2014). As a whole, these data suggest that HcrtR1 antagonists might be of potential interest for the treatment of drug addiction (Figure 3). The possible usefulness of HcrtR2 antagonists should be considered cautiously since this receptor seems to be mainly involved in the regulation of sleep-wake cycle.

Neurobiological mechanisms of cannabinoid dependence

The neurochemical processes by which cannabinoid addiction is developed are similar to those reported for other drugs of abuse. The endocannabinoid system is the leading site of action for the rewarding and pharmacological responses induced by cannabinoids. In fact, this system fulfils a general modulatory effect on the reward circuitry, participating in the rewarding and addictive properties of all classical drugs of abuse.

CB1 receptors are abundantly expressed in the diverse regions of the brain reward system, including the VTA, the NAc, the prefrontal cortex and the amygdala. However, it is known that VTA dopaminergic neurons are unlikely to express CB1 receptors (Julian et al 2003). Indeed, CB1 receptors present in the VTA are located on presynaptic glutamatergic and GABAergic neurons. Endocannabinoids modulate the excitatory and inhibitory synaptic inputs into the VTA acting as a retrograde messenger. Thus, the activation of CB1 receptors in the VTA present in GABAergic interneurons or in glutamatergic terminals mainly from prefrontal cortex neurons would remove, respectively, these inhibitory or excitatory inputs on dopaminergic neurons. The final effect on the modulation of VTA dopaminergic activity by endocannabinoids would depend on the functional balance between these GABAergic and glutamatergic inputs (Maldonado et al 2006; Fattore et al 2008). Accordingly, exogenous cannabinoid agonists stimulate the activity of mesencephalic dopaminergic neurons by altering this balance. Both Δ^9-tetrahydrocannabinol (THC) and the synthetic cannabinoid WIN55,212-2 enhance the firing rate and bursting activity of dopaminergic neurons in the VTA, subsequently enhancing dopamine release in terminal regions such as the NAc and the

prefrontal cortex (Chen et al 1990; Tanda et al 1997), a fact that has been associated to their reinforcing properties.

Parallel dopamine-independent mechanisms in the development of cannabinoid addiction may also be modulated by the endocannabinoid system. For instance, CB1 receptors present in the prefrontal cortex might explain the involvement of the endocannabinoid system in the motivation to seek the drug since this brain area integrates sensory information, emotional processing and hedonic experience. Besides, reinstatement of drug-seeking behaviour is probably influenced by the endocannabinoid system because of its capacity to modulate synaptic plasticity, consolidating the reward-driven behaviour required to establish addictive processes (Maldonado et al 2006).

In summary, endocannabinoid and dopaminergic systems are crucially involved in the neurobiological mechanisms underlying the addictive effects of the main classical drugs of abuse, including cannabinoids. Likewise, other neurochemical systems have also been involved in the addictive processes induced by cannabinoids, including endogenous opioids, GABA, glutamate, monoamines and several neuropeptides (Maldonado et al 2011). Lately, hypocretins have been reported to contribute also to cannabinoid dependence, revealing a new potential therapeutic target for the treatment of this disorder.

Interaction between hypocretin/orexin and endocannabinoid systems

In the latest years research has shown the possible existence of a cross-talk between hypocretinergic and endocannabinoid systems. Although few investigations have analysed this interaction, emerging neuroanatomical and biochemical evidences strongly suggest the existence of such cross-modulation (Flores et al 2013). Hence, CB1 and HcrtRs show an overlapping distribution in several areas of the CNS (Marcus et al 2001; Mackie, 2005). The common expression of HcrtRs and CB1 within the entire hypothalamus denotes an important modulation of energy homeostasis and neuroendocrine and autonomic functions by both hypocretin and endocannabinoid systems. They are also abundant along

the mesocorticolimbic system, denoting their regulation of reward processing and addiction. Likewise, the mutual involvement of these neuromodulators in the control of anxiety-like responses, sleep/wake cycle and nociception is supported by the presence of HcrtRs and CB1 within diverse brainstem nuclei. However, HcrtRs location among different neuronal populations and therefore direct synaptic connections between HcrtRs and CB1 are not well defined yet. On the other hand, the recent detection of multifocal CB2 expression in the brain opens a new range of possibilities for hypocretin-cannabinoid interaction.

CB1 and CB2, as well as HcrtR1 and HcrtR2, are classified as rhodopsin-like G-protein coupled receptors. Although most cellular signals triggered upon cannabinoid receptor activation differ from those initiated following the stimulation of hypocretin receptor, it seems that cannabinoid and HcrtRs share diverse signaling pathways (Demuth & Molleman, 2006; Kukkonen, 2013). CB1 and CB2 receptors are coupled to the Gi/o family of G-proteins and subsequently inhibit adenylyl cyclase activity and decrease cAMP levels. In contrast, HcrtRs are mainly associated with Gq-proteins, inducing the activation of phospholipase C which produces the second messengers DAG and IP3. Additionally, stimulation of both HcrtRs has been suggested to modulate adenylyl cyclase activity by coupling other G-proteins in certain experimental conditions. One of the most relevant differences between hypocretin and cannabinoid cellular signaling is the effect on transmembrane potential as a consequence of their divergent modulation of effector ion channels triggered upon receptor stimulation. Thus, CB1 activation triggers the plasmatic membrane repolarization which results in neurotransmitter release inhibition, whereas HcrtRs stimulation induces membrane depolarization facilitating the formation of action potentials. On the other hand, the main cellular pathway activated by both cannabinoid and hypocretinergic stimulation is the phosphorilation and activation of the MAP kinase cascade, which regulates neuronal gene expression and synaptic plasticity. Additionally, recent *in vitro* and *in vivo* studies report that HcrtR1 stimulation can lead to 2-arachidonoylglycerol (2-AG) release activating CB1 receptors in nearby cells, since DAG

produced upon phospholipase C stimulation is used by diacylglycerol lipase as a substrate for 2-AG synthesis (Turunen et al 2012). This hypocretin-induced endocannabinoid release might be a relevant mechanism by which hypocretins could mediate synaptic inhibition in certain conditions.

CB1 receptors have a significant propensity to make homo- and heteromeric complexes. Indeed, the existence of CB1-HcrtR1 and CB1-HcrtR2 heteromers in diverse *in vitro* models has been demonstrated by electron microscopy colocalization and fluorescence and bioluminiscence resonance energy transfer (FRET and BRET) imaging studies (Hilairet et al 2003; Ellis et al 2006). The functional impact of this heteromerization has been associated with HcrtRs and CB1 receptor traficking as well as cross-agonism/-antagonism phenomena. However, whether HcrtR-CB1 heteromers actually exist within the CNS tissue and if they are indeed of physiological relevance remain to be further elucidated.

So far various functional studies have revealed the existence of a mutual regulation between the hypocretin and endocannabinoid systems, mainly regarding the areas of nociception, appetite and reward. Thus, systemic administration of the CB1 antagonist AM251 reverses the antinociceptive effect of hypocretin-1 microinjection into the periaqueductal gray during the hot-plate test in rats (Ho et al 2011). Similarly, it has been suggested that hypocretin-1 exerts its orexigenic action through CB1 receptor activation, since pre-treatment with a non-anorectic dose of the CB1 antagonist rimonabant blocks the orexigenic effect of hypocretin-1 administered by intracerebroventricular route in rats (Crespo et al 2008). In the same line, it has been shown that conditioned place preference induced by chemical stimulation of the LH, which depends on HcrtR1 activation in the VTA, is reduced in a dose-dependent manner by previous intra-VTA administration of rimonabant (Taslimi et al 2011). Moreover, co-administration of effective doses of both HcrtR1 and CB1 antagonists into the VTA reduces conditioned place preference in a non-additive manner, suggesting that these receptors regulate this effect by a common mechanism (Taslimi et al 2011). This goes in line with the idea that hypocretins and endocannabinoids may share a common site of action for

reward modulation, since primary rewarding effects of drugs of abuse that depend on increased VTA dopaminergic activity, such as opioids, nicotine and alcohol, require both hypocretinergic transmission and presence of CB1 receptors. A possible explanation for this cannabinoid-mediated effects of hypocretins is the synthesis and release of 2-AG upon HcrtR1 activation mentioned above. However, this mechanism has been only confirmed to occur in the periaqueductal gray (Ho et al 2011) and in the dorsal raphe nucleus (Haj-Dahmane & Shen, 2005) and further studies will be necessary to better understand the interaction between the endocannabinoid and hypocretin systems in the regulation of the reward circuit.

Role of hypocretins/orexins in cannabinoid dependence

Despite the evidence supporting the hypocretin-cannabinoid cross-talk in the CNS and the undeniable regulation of the addictive properties of several drugs of abuse by the hypocretinergic system, there is little information regarding the role of hypocretins in cannabinoid dependence. The main data available are focused on the rewarding properties of cannabinoids, whereas other relevant aspects of cannabinoid dependence such as relapse and withdrawal remain unexplored.

The initiation of cannabis addiction has been related to its capacity to induce rewarding effects. There are several predictive animal models to study responses related to the rewarding effects produced by cannabinoids. Among these paradigms, drug self-administration procedures currently represent the most reliable models of drug consumption in humans and have a high predictive value by directly evaluating the reinforcing properties of the drug (Maldonado et al 2011). Recent studies have revealed persistent cannabinoid self-administration under different experimental conditions. Thus, intravenous self-administration of THC has been described in squirrel monkeys (Justinová et al 2003). Although operant responding for self-infused THC has not been consistently demonstrated in rodents, intravenous self-administration of

the synthetic and short half-life cannabinoid agonist WIN55,212-2 has been reported in rats and mice (Solinas et al 2007; Mendizábal et al 2006).

A recent study has investigated the contribution of hypocretins to the reinforcing properties of cannabinoids by using the intravenous WIN55,212-2 self-administration paradigm in mice (Flores et al 2014). In this study animals were trained to self-administer the synthetic cannabinoid during 12 days and the role of HcrtRs was evaluated by diverse pharmacological and genetic approaches (Figure 4). The study showed that a chronic treatment with the HcrtR1 antagonist SB334867 during the acquisition phase of WIN55,212-2 self-administration reduced the number of animals reaching the acquisition criteria for this operant response. Moreover, acute SB334867 administration after a successful acquisition of WIN55,212-2 self-administration impaired the reinforcing properties of this synthetic cannabinoid. An acute HcrtR1 antagonist injection also reduced the maximal effort required to obtain a WIN55,212-2 infusion in a progressive ratio schedule session, suggesting a decrease in the motivation for the drug. On the contrary, the administration of the HcrtR2 antagonist TCSOX229 did not modify WIN55,212-2 self-administration in any of the reinforcement schedules tested. These data point to a specific role for HcrtR1 in the modulation of the reinforcing properties of cannabinoids. In fact, HcrtR2 appears to be of minor relevance in the regulation of the rewarding properties of drugs of abuse, although both hypocretin receptor subtypes are abundant in brain areas relevant in reward processing, such as the VTA, NAc and bed nucleus of the stria terminalis (Plaza-Zabala et al 2012). In agreement with the pharmacological data previously mentioned, HcrtR1 knockout mice showed an impaired WIN55,212-2 self-administration behaviour in comparison with wild-type animals (Flores et al 2014). Thus, the acquisition of the operant behaviour was successfully reached by a minor percentage of mutant mice. The amount of WIN55,212-2 self-infused by these knockout animals was reduced as well. Mice lacking HcrtR1 also showed diminished motivation for the cannabinoid, since their maximal effort to obtain a WIN55,212-2 infusion was reduced when compared to wild-type

mice in a progressive ratio schedule session. It is worth mentioning that the impairment of WIN55,212-2 self-administration was not attributable to other unspecific effects such as locomotion alteration or possible learning deficits in the acquisition of an operant behaviour, since none of the treatments or genetic manipulations altered operant responding for a natural reward such as water. As a whole, these behavioural data suggest that the primary reinforcing properties of cannabinoids as well as motivation to obtain the drug are modulated by HcrtR1 signaling (Flores et al 2014).

Little information is available regarding the possible changes of the hypocretinergic activity in response to a chronic cannabinoid exposure. It has been recently shown that chronic THC administration during the adolescent period of male rats decreases hypothalamic prepro-hypocretin mRNA levels in adulthood (Llorente-Berzal et al 2013). Interestingly, hypothalamic prepro-hypocretin mRNA levels remain unaffected in adult female rats receiving THC during adolescence (Llorente-Berzal et al 2013). This sexual dimorphism results interesting since female rats also acquire stable WIN55,212-2 self-administration more rapidly and at higher rates than males (Fattore et al 2007). Thus, lower hypocretin levels could be related to phenotypes less vulnerable to cannabinoid dependence. On the other hand, it has been observed that hypocretin-1 expression in peripheral blood cells is reduced in cannabis-dependent smokers when compared to nicotine-dependent smokers and non-smokers (Rotter et al 2012). However, this information should be carefully interpreted, since peripheral hypocretin mRNA levels do not necessarily reflect the situation in certain areas of the CNS. Moreover, these differences could be related to peripheral effects of THC and not to the central modulation of cannabinoid dependence (Flores et al 2013).

Operant yoked-control models of drug self-administration are essential to understanding whether neurochemical changes are attributable to the effects of drug seeking or to the pharmacologic actions of the drug. Interestingly, contingent (active) and non-contingent (passive) WIN55,212-2 self-administration have been reported to differently modulate the

activation of hypocretin neurons (Flores et al 2014). Thus, immunofluorescence studies showed that contingent self-administration of the cannabinoid induced an increase in the percentage of hypocretin cells expressing FosB/ΔFosB in the LH, a transcription factor which is considered a marker of neuronal activity (Figure 5). Notably, hypocretin cells were not activated in mice receiving passive infusions of the cannabinoid, suggesting that the recruitment of hypocretin cells within the LH is mainly due to operant seeking for the reinforcing effects of this drug. Moreover, these neurons were not recruited due to the learning process inherent to the acquisition of an operant behaviour since operant conditioning maintained by water did not modify the activation of hypocretin cells within the LH. In contrast, operant WIN55,212-2 self-administration had no apparent effect on FosB/ΔFosB expression in DMH/PFA hypocretin neurons, although mice receiving passive infusions of the drug showed a lower activation of these cells in comparison with those receiving the drug contingently (Figure 5). Activation of hypocretin cells located within the LH, but not those in the DMH/PFA, has been related also with the exposure to other drugs of abuse such as morphine (Richardson & Aston-Jones, 2012) and ethanol (Lawrence et al 2006). In fact, it has been proposed the existence of a functional dichotomy between the diverse hypocretin neurons subpopulations, being those located in the LH mainly involved in reward processing whereas those located in the DMH/PFA more related with regulation of arousal and stress (Harris & Aston-Jones, 2006). However, some divergent findings suggest that further studies are required to support a differentiated role for each hypocretin cell subpopulation.

As previously mentioned, the modulation of VTA dopaminergic transmission by hypocretins seems to be especially important in the regulation of reward processing, particularly in drug seeking behaviour (Mahler et al 2012). Accordingly, cannabinoid-induced release of dopamine in the NAc, which correlates with the rewarding effects of the drug, seems to be modulated by HcrtR1 signaling. Thus, increased extracellular dopamine levels in the NAc induced by an acute systemic injection of THC was observed in wild-type mice but not in HcrtR1 knockout animals, as

revealed by in vivo microdialysis studies (Figure 6) (Flores et al 2014). Although the neuronal mechanism remains unclear at present, it seems that cannabinoid exposure could activate hypocretin transmission in the VTA. The subsequent activation of HcrtR1 in the VTA might be contributing to the rewarding properties of cannabinoids through the modulation of the dopamine mesolimbic pathway.

In summary, hypocretin transmission at HcrtR1 is a critical component involved in the reinforcing and motivational properties of cannabinoids. Therefore, HcrtR1 antagonists could represent an interesting pharmacologic tool for cannabis dependence in humans, which is a major clinical need devoid of available treatment at the present moment.

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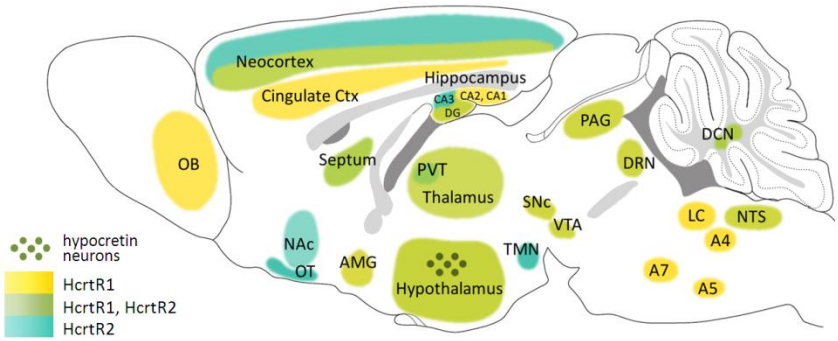
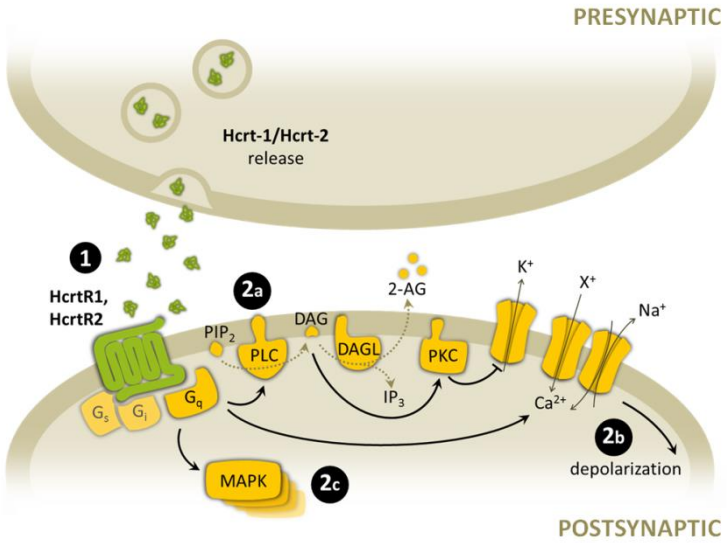
Figure 1.



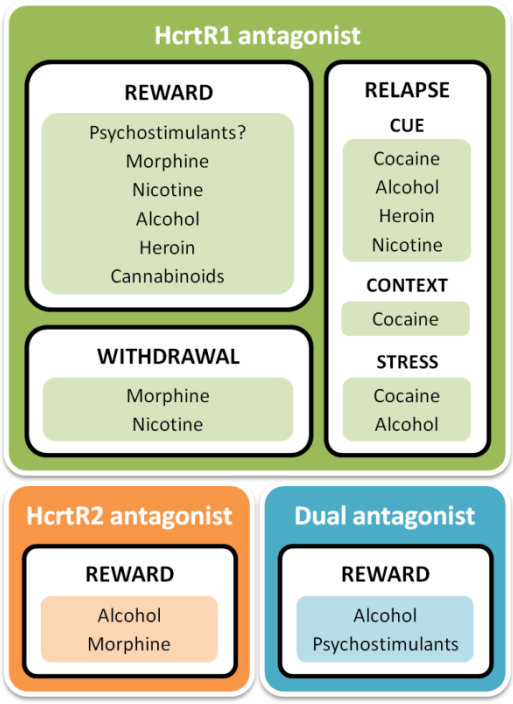
Hypocretinergic/orexinergic system distribution in the brain. Schematic representation of the main areas expressing HcrtR1 and HcrtR2 in the mouse brain and location of hypocretin/orexin neurons. A4, A5, A7, pons cell groups; AMG, amygdala; Ctx, cortex; DCN, deep cerebellar nuclei; DG, dentate gyrus; DRN, dorsal raphe nucleus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; PVT, paraventricular nucleus of thalamus; SNc, substantia nigra pars compacta; TMN, tuberomammillary nucleus; VTA, ventral tegmental area. Adapted with permission from Flores Á (2013) Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far. Front Neurosci, 7:256. doi:10.3389/fnins.2013.00256.

Figure 2.



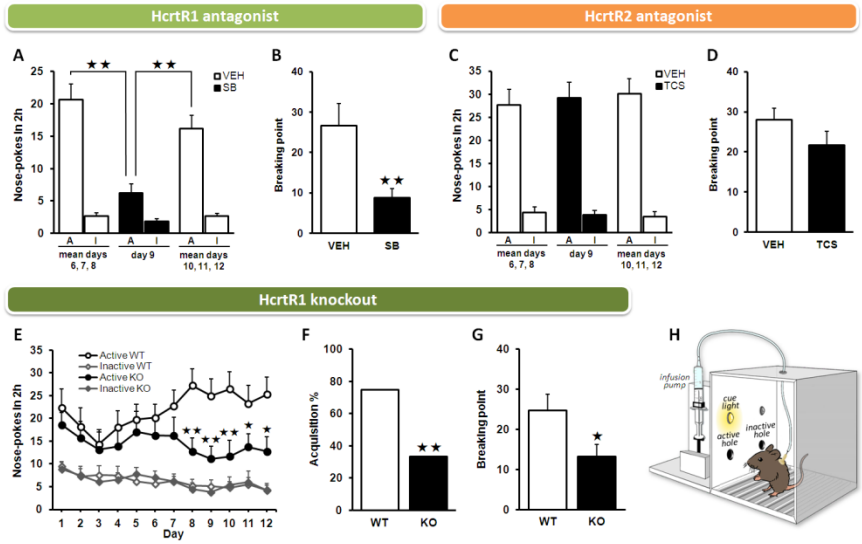
Hypocretin-mediated synaptic signaling. (1) Hypocretins are released from presynaptic terminals and stimulate postsynaptic HcrtR1 and HcrtR2. (2) HcrtR activation is mainly associated with Gq-protein stimulation, but it can activate also other G-protein subtypes. The key downstream outcomes from HcrtR activation and subsequent Gq-protein stimulation are: (2a) activation of PLC activity, and preceding DAG and 2-AG synthesis (2b) membrane depolarization due to the modulation of K+ channels, non-specific cationic channels and Na+/Ca2+exchanger, and (2c) activation of protein kinase cascades such as the MAPK pathway. PIP2, phosphatidylinositol bisphosphate; DAG, diacylglicerol; 2-AG, 2-arachidonoylglycerol; PLC, phospholipase C; DAGL, diacylglycerol lipase; MAPK, mitogen-activated protein kinase; Hcrt-1, hypocretin-1; Hcrt-2, hypocretin-2; PKC, protein kinase C; X+, unspecific cation. Adapted with permission from Flores Á (2013) Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far. Front Neurosci, 7:256. doi:10.3389/fnins.2013.00256.

Figure 3.



Potential therapeutic utility of hypocretin/orexin receptor antagonists. Diagram showing the different stages of drug addiction in which hypocretin/orexin receptor antagonists constitute potential therapeutic tools. Adapted with permission from Plaza-Zabala A (2012) The hypocretin/orexin system: implications for drug reward and relapse. Mol Neurobiol, 45(3):424. doi: 10.1007/s12035-012-8255-z.

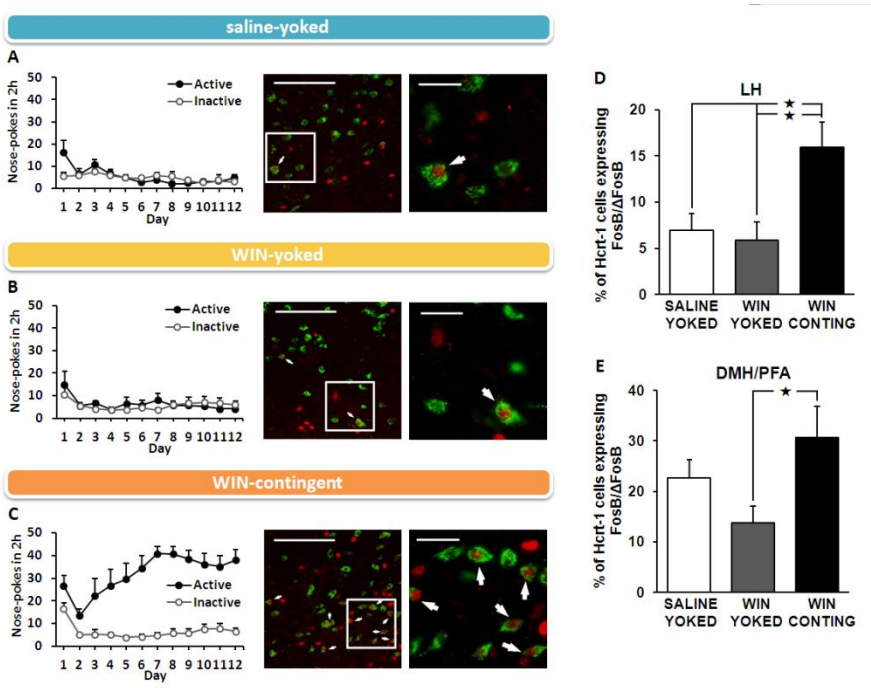
Figure 4.



Effects of HcrtR1 and HcrtR2 pharmacological blockade or HcrtR1 gene deletion in WIN55,212-2 self-administration. HcrtR1 antagonization or gene deletion reduces operant WIN55,212-2 self-administration and motivation to obtain the drug, whereas HcrtR2 pharmacological blockade has no effect on this behavioural response. In the pharmacological approach (A-D) mice were trained to self-administer the synthetic cannabinoid WIN55,212-2 under fixed ratio 1 (FR1) until stable behaviour was acquired. SB334867 (10 mg/kg, intraperitoneal) or TCSOX229 (10 mg/kg, intraperitoneal) was administered 30 minutes before a FR1 session on day 9 or before the progressive ratio (PR) session. (A,C) Number of active and inactive responses displayed after acute (A) SB334867 or (C) TCSOX229 pre-treatment on day 9 and during the 3 days before (mean days 6–8) and after (mean days 10–12) these pharmacologic challenges. (B,D) Breaking-point values achieved in the PR schedule after acute (B) SB334867 or (D) TCSOX229 pre-treatment. In the genetic approach (E-G) HcrtR1 knockout and wild-type mice were trained to self-administer WIN55,212-2 during 12 days and underwent a PR session on day 13. The time course (E) and the percentage of acquisition (F) of WIN55,212-2 self-administration in wild-type and knockout mice is shown, as well as the breaking-point values

achieved in the PR schedule (G). (H) Schematic diagram of operant chambers where drug self-administration procedures are performed. Values are represented as mean+SEM. SB, SB334867; VEH, vehicle; TCS, TCSOX229; WT, wild-type; KO, HcrtR1 knockout; A, active hole; I, inactive hole. Adapted with permission from Flores Á (2014) The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. Biol Psychiatry, 75(6):499. doi: 10.1016/j.biopsych.2013.06.012.

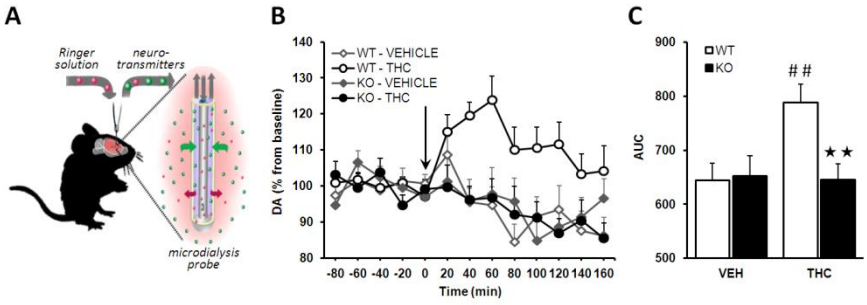
Figure 5.



Hypocretin/orexin neurons in the lateral hypothalamus are activated during contingent, but not noncontingent, WIN55,212-2 self-administration. Double-label immunofluorescence of FosB/ΔFosB with hypocretin-1 was performed in the lateral as well as dorsomedial and perifornical hypothalamic area of mice that had underwent a contingent/noncontingent WIN55,212-2 self-administration paradigm during 12 days. (A–C) Operant responding on the active and inactive holes in mice receiving (A) passive saline infusions (saline-yoked mice), (B) passive intravenous WIN55,212-2 infusions (WIN-yoked mice), and (C) contingent intravenous WIN55,212-2 infusions (WIN-contingent). Each group is illustrated with their corresponding representative images of sections of the LH obtained via fluorescence microscopy. Arrowheads indicate FosB/ΔFosB positive hypocretin-1 expressing neurons. The scale bar represents 100 µm in left-side images and 25 µm in right-side images. (D,E) Percentage of FosB/ΔFosB positive hypocretin-1 expressing neurons after contingent/noncontingent WIN55,212-2 self-administration in the LH

(D) and the DMH/PFA (E). Values are represented as mean+SEM. LH, lateral hypothalamus; DMH/PFA, dorsomedial and perifornical area; WIN, WIN55,212-2. Adapted with permission from Flores Á (2014) The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. Biol Psychiatry, 75(6):499. doi: 10.1016/j.biopsych.2013.06.012.

Figure 6.



THC-induced enhancement in dopamine extracellular levels in the nucleus accumbens is blocked in HcrtR1 knockout mice. (A) Schematic diagram of *in vivo* microdialysis principles. (B) Time course of basal and stimulated levels of dopamine (percentage from baseline) in the nucleus accumbens. Dialysates were collected 80 minutes before and 160 minutes after a challenge injection of THC (0.3 mg/kg, intraperitoneal; arrow at time 0) or vehicle in wild-type and HcrtR1 knockout mice. (C) Area under the curve (AUC) values for levels after THC or vehicle injection (from 0 to 160 minutes) for the different groups of mice. Values are represented as mean+SEM. DA, dopamine; WT, wild-type; KO, knockout; VEH, vehicle. Adapted with permission from Flores Á (2014) The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. Biol Psychiatry, 75(6):499. doi: 10.1016/j.biopsych.2013.06.012.

MINI-DICTIONARY OF TERMS

Reinforcement: it is a term used in operant conditioning to refer to a stimulus that increases the probability that a response will occur. For instance, in an attempt to increase the likelihood of a behaviour occurring in the future, an operant response is associated by the presentation of a reward.

Synaptic plasticity: group of processes that confer to a synapse the ability to strengthen or weaken its capacity of transmission.

Mesocorticolimbic system: group of brain structures involved in the processing of reward and hedonic experiences, including the ventral tegmental area in the brainstem and its target structures, such as nucleus accumbens, prefrontal cortex, amygdala and bed nucleus of stria terminalis.

G-protein coupled receptor: receptor located in the cellular membrane which activates an intracellular pathway through stimulation of a G protein.

Heteromer: a complex formed by different types of subunits. In this case, this complex is formed by diverse receptors, and shows features distinct from those of its components.

Fluorescence and bioluminescence resonance energy transfer (FRET and BRET): *in vitro* technique used to determine if two components are close enough to form a complex, based in the distance at which different types of energy is able to be transferred from one to another.

Conditioned place preference: behavioural paradigm used to determine the rewarding or aversive effects of a drug, performed in a two-compartment box. Mice are administered with a drug in one of the compartments and with vehicle in the other compartment during several days. The time spent in each compartment during the test day gives an idea of the reward or aversion induced by the drug.

***In vivo* microdialysis**: technique used to measure *in vivo* the amount of a determined compound (e.g. a neurotransmitter) released to the extracellular space in a small volume of tissue (e.g. a defined brain structure). It requires the implantation of a probe containing a semipermeable membrane, which will be crossed by small solutes by passive diffusion.

KEY FACTS OF DRUG SELF-ADMINISTRATION PARADIGMS

- ✓ Drug self-administration is a form of operant conditioning used for assessing drug-seeking and drug-taking behaviour.
- ✓ This method allows us to directly evaluate the reinforcing properties of a drug, and hence to predict its rewarding (and presumably addictive) effects.
- ✓ Drug self-administration protocols are performed in operant conditioning chambers, generally equipped with two levers (or holes), one active and one inactive, distinguishable for the presence of a cue light above the active one.
- ✓ A correct lever-pressing or nose-poking response results in the contingent presentation of reward: the delivery of an intravenous drug infusion by a syringe pump.
- ✓ Different reinforcement schedules can be employed depending on the type of response to be evaluated:

  - Fixed-Ratio (FR). A determined number of operant responses are required to dispense one unit of reinforcer, i.e. FR5 requires 5 active lever-presses to receive one drug infusion. Commonly used during the acquisition of drug-taking.

  - Progressive Ratio (PR). The number of operant responses required to receive one reinforcer are gradually increased. This schedule provides information about the motivation to obtain the drug, since it reveals at which amount of effort the subject ceases drug self-administration.

- ✓ Drug self-administration allows the study of diverse stages present in the addictive behaviour:

  - Initial acquisition of drug-taking behaviour

  - Extinction of this behaviour during the absence of the drug

  - Reinstatement of drug-seeking after exposure to stress, associated context/cues, or the drug itself

SUMMARY POINTS

- Hypocretins/orexins are hypothalamic neuropeptides which regulate physiological processes such as energy balance, stress and arousal, sleep/wake cycle, and reward.
- The hypocretinergic system modulates the addictive properties of several drugs of abuse (i.e. alcohol, nicotine, opioids, psychostimulants and cannabinoids).
- Anatomical, biochemical and functional cross-talks exist between hypocretinergic and endocannabinoid systems, being the last one the main target of cannabis compounds.
- The hypocretin receptor 1 is necessary for the rewarding properties of the synthetic cannabinoid WIN55,212-2.
- Chronic exposure to THC or WIN55,212-2 affects the activity of hypocretin neurons.
- The dopaminergic transmission in the mesolimbic system is disrupted if the hypocretin receptor 1 is not present.