DOLOR Y RECAÍDA EN EL ALCOHOLISMO: ALTERACIONES EN LA FUNCIÓN MODULADORA DEL SISTEMA OPIOIDE ENDÓGENO SOBRE EL SISTEMA DOPAMINÉRGICO MESOCORTICOLÍMBICO

TESIS DOCTORAL

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CERTIFIQUEN

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València, 29 de gener de 2020

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A Lucía, Ana y Luis

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ABBREVIATIONS

ACC	Anterior cingulate cortex
ACD	Acetaldehyde
AMPAR	**α**-amino-3-hydroxi-5-methyl-4-isoxazole propionic acid receptor
AUD	Alcohol use disorder
BNST	Bed nucleus of the stria terminalis
CeA	Central amygdala
CNS	Central nervous system
CPP	Conditioned place preference
CRF	Corticotropin releasing factor
D1	DA type 1 receptor
DA	Dopamine
DLPT	Dorsolateral pontine tegmentum
DOR	delta-opioid receptor
DRG	Dorsal Root Ganglia
EOS	Endogenous opioid system
GPCR	G protein-coupled receptor
IL	Infralimbic cortex
KO	Knockout
KOR	kappa-opioid receptor

LTP	Long-term potentiation
MCLS	Mesocorticolimbic system
MOR	mu-opioid receptor
NAc	Nucleus accumbens
NMDAR	N-methlyd-D-aspartate receptor
OR	Opioid receptor
ORL1	Opioid receptor like-1
PAG	Periaqueductal gray
PBP	Parabrachial pigmented nucleus
PFC	Prefrontal cortex
PL	Prelimbic cortex
PN	Paranigral nucleus
RVM	Rostral ventromedial medulla
SAL	Salsolinol
SN	Substantia nigra
SUD	Substance use disorder
TH	Tyrosine hydroxylase
THIQ	Tetrahydroisoquinoline
VP	Ventral pallidum
VTA	Ventral tegmental area

1 INTRODUCTION

The latest "Status Report in Alcohol and health" from the World Health Organization (WHO) Regional Office of Europe reveals that **Alcohol Use Disorder (AUD)** is the **third leading risk of burden of disease** in Europe and is very resistant to pharmacological or other kinds of treatment, specially in certain clinical situations. The WHO Regional Office for Europe points to the need of more research effort to clarify which are these special clinical situations that increase the risk of suffering AUD.

On the other hand, chronic pain is a major health problem affecting to 20-30% of the general European population. Notably, persistent pain conditions are often accompanied by comorbid affective and emotional disorders, which are very difficult to treat. Recent human and animal studies revealed that **pain negatively impacts on the motivational and reward processing**, via altering the normal function of the mesocorticolimbic system (MCLS). This system is responsible for the expression of motivated behavior and reinforcement learning triggered by natural and drug rewards and also for aversive stimuli encoding. Therefore, the effects of persistent pain in the MCLS not only might affect the quality of life of patients (e.g., provoking anhedonia, depression, negative affect state), but also it might have an important impact on vulnerability to drug abuse.

Multiple clinical and epidemiological studies have revealed that the presence of **chronic pain is closely related to AUD**. All this evidence points to chronic pain as a factor increasing the risk of suffering AUD, predicting heavy drinking behavior and relapse in those patients with a previous history of alcohol abuse. However, and despite the clinical relevance of this evidence in the selection and design of pain and AUD treatment strategies, few studies have examined the neural mechanisms and circuits underlying this relationship.

All efforts to reduce AUD prevalence are needed, including research in understanding the factors promoting heavy drinking and relapse. Specially, during the last years research has focused on finding new strategies for the prevention of relapse, as it constitutes the main clinical problem in alcohol-dependent patients. The **better understanding of the possible pain-induced effects on AUD and relapse will lead to relevant results** with an extremely applicability in the early detection and prevention of AUD in pain patients. Moreover, it should also help to the development of novel and safer therapeutic strategies for treating pain and AUD.

The present Thesis is devoted to explore the influence of pain on the modulatory role of the mu opioid receptor (MOR) on the MCLS and the possible pain-induced effect on the motivational properties of alcohol. In addition, this Thesis also shows relevant data regarding the effect of pain on alcohol relapse.

1.1 MCLS: A KEY BRAIN NETWORK IN ADDICTION

Drug addiction is a very complex pathology that encompasses different brain regions involved in reward, emotion, decision making and habit. It is now widely accepted that all drugs of abuse are reinforcing, that is, they increase the likelihood of responses that produce them (Everitt and Robins 2015). Their reinforcing properties are mediated by the drug action on the MCLS. This system, that is normally activated by natural reinforces such as food, water or sex, is composed by a set of interconnected forebrain structures. In this way, the MCLS is a heterogeneous brain network that encompasses not only several structures but also a diversity of neurotransmitters. Concretely, those structures include the nucleus accumbens (NAc) (the major component of the ventral striatum), the extended amygdala (NAc shell, bed nucleus of the stria terminalis (BNST) and central amygdala (CeA)), hippocampus, hypothalamus, and frontal regions of the cerebral cortex. All this regions receive dopaminergic innervation from the ventral tegmental area (VTA) of the midbrain (reviewed in Fields et al., 2007). The dopamine (DA) neurons are key elements in the function of the MCLS; however, other neuron types and neurotransmitters also play a modulatory role. A simplified representation of the structures that constitute the MCLS can be found in Figure 1.1.

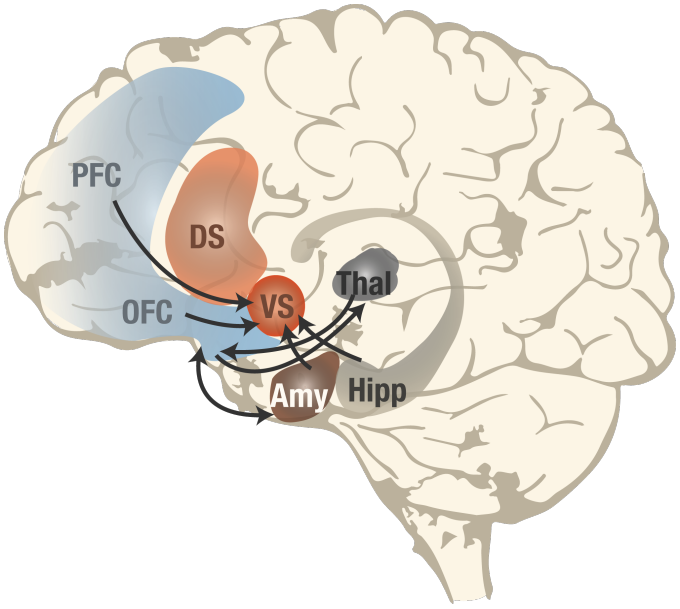

Figure 1.1 Neural reward circuits implicated in drug addiction (including ethanol). The main important brain areas involved in drug addiction and their main connections (black arrows) are represented in the picture (adapted from Everitt and Robbins 2005). Abbreviations: Amy, amygdala; Hipp, hippocampus; Thal, thalamus; DS, dorsal striatum, VS, ventral striatum; OFC, orbitofrontal cortex; mPFC, medial prefrontal cortex.

Under normal circumstances, natural reinforcers activate DA neurons in the MCLS and consequently DA release is enhanced. This mechanism is the key to produce the reinforcement of behaviors that are necessary for survival and reproduction (Fields et al., 2007). Drugs of abuse overstimulate the same DA circuits, what constitutes the initial step for the development of addiction disorders (a detailed review of the different phases of addiction can be found in Koob and Le Moal 2006). Given the important role of the neurotransmitter DA and the DA neurons in the MCLS in reinforcing behaviors and addiction disorders, a more detailed description of this group of neurons will be provided in section 1.1.

The **VTA** is the midbrain region that **includes dopaminergic neurons** of the A10 cell group (Dahlstroem and Fuxe 1964). It is situated medial and contiguous to the substantia nigra (SN), immediately ventral to the red nucleus and caudal to the hypothalamus (Björklund and Dunnett 2007). Based on the heterogeneity of the morphology and orientation of cells, the VTA can be subdivided in five regions: two lateral nuclei (the paranigral nucleus (PN) and the parabrachial pigmented nucleus (PBP)) and three median nuclei (the interfascicular, the rostral linear and the caudal linear nuclei) (for a recent review of the VTA anatomy see Sánchez-Catalán et al., 2014). This definition slightly differs from the original description by Tsai (Tsai 1925) that did not include midline nuclei; however, it is widely accepted that all five nuclei include DA neurons from the A10 cathecolaminergic group (Sánchez-Catalán et al., 2014).

Traditionally, VTA DA midbrain neurons have been identified by pharmacological and electrophysiological criteria, considering them as a homogeneous and well characterized population. Concretely, the identification of putative DA cells by electrophysiological means has been based on low-frequency pacemaker activity, broad action potentials, hyperpolarization by DA via D_2 receptors or the presence of the so-called hyperpolarization-activated slow inward current (Ih current) (Kitai et al., 1999; Lammel 2014). However, several studies have found neurons immunohistochemically defined as DA (i.e., identified tyrosine hydroxylase (TH) positive) among this area that do not share these same properties (Lammel et al., 2008; Margolis et al., 2006; 2008; Nair-Roberts et al., 2008; Swanson 1982; Ungless and Grace 2012). These studies have shown that **VTA DA neurons constitute a heterogeneous population** with variable molecular and electrophysiological properties depending on their projecting area (Lammel et al., 2008; Margolis et al.,

2008). In fact, some authors have suggested that VTA DA neurons should be classified into two different subpopulations (Lammel et al., 2014), consisting in: (i) DA neurons mainly located in the lateral posterior and anterior VTA (lateral PBP), that project to the NAc lateral shell. This subpopulation would exhibit the electrophysiological properties that were classically attributed to DA neurons such as a low firing frequency and a large Ih current (Lammel et al., 2008; Lammel et al., 2014; Ungless and Grace 2012; Zhang et al., 2010). Functionally, DA neurons projecting to NAc lateral shell seem to be particularly involved in reward-related behaviors (Lammel et al., 2012; Morales et al., 2017). And (ii), a later identified second subpopulation of DA neurons localized in the medial posterior VTA (PN and medial PBP) that project to the prefrontal cortex (PFC), BLA and the NAc medial shell and core. These neurons, immunohistochemically defined as DA, showed a "non-conventional" electrophysiological profile characterized by a small Ih current and a high firing frequency (Lammel et al., 2014). In this case, the functional implication of these neurons in reward or aversive behaviors appears to be more complex and is also determined by the different neuronal inputs and outputs of these DA neurons (Bariselli et al., 2016; Morales et al., 2017).

As pointed out, the VTA has been classically defined as a dopaminergic region; although, in the rat brain, only around **60%** of its containing neurons are **DA neurons** (Swanson 1982; Margolis et al., 2006; Nair-Roberts 2008). The majority of the resting neural population (around **30%**) can be cytochemically identified to **GABA neurons** (Carr and Sesack 2000, Margolis et al. 2006, Van Bockstaele and Pickel 1995). There is also evidence that **2-3%** of the neurons release glutamate (**Glu neurons**) (Chuhma et al., 2004; Lavin et al., 2005). It is also important to consider that many types of neurons, including DA, release more than one neurotransmitter. In this way, recent

optogenetic studies have shown that DA neurons can release glutamate and GABA to varying extents, in addition to DA (reviewed in Britt and Bonci 2013).

The activity of DA neurons in the VTA is mainly under the control of GABAergic and glutamatergic afferents originating in very different brain areas. Following, the main afferents that arrive into the VTA, as well as the different VTA outputs have been briefly described. Also Figure 1.2 shows an extremely simplified scheme of the neural organization of the VTA, including the main inputs that regulate the activity of the DA neurons and the main projection areas.

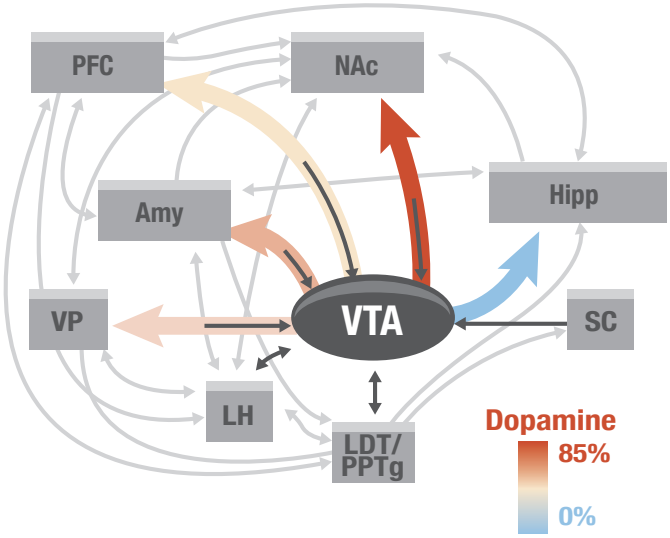

Figure 1.2 Schematic representation of the neural organization of the VTA. The projections from and to the VTA are represented in color (different in base to the fibers content in DA) and in black. Other connections are represented in grey (adapted from Fields et al. 2007). Abbreviations: Amy, amygdala; Hipp, hippocampus; LH, lateral hypothalamus; LDT, laterodorsal tegmental nucleus; NAc, nucleus accumbens;, PPTg, pedunculopontine tegmental nucleus; SC, superior colliculus; VP, ventral pallidum.

1.1.1 VTA INPUTS

The VTA receives projections from multiple brain regions (Geisler and Zahm 2005; Phillipson 1979; Yetnikoff et al., 2014).

It receives excitatory **glutamatergic** inputs from:

- PFC (Sesack and Pickel 1992)
- Lateral hypothalamus (Rosin et al., 2003)
- BNST (Georges and Aston-Jones 2002)
- Superior colliculus (Geisler and Zahm 2005)
- Mesopontine tegmental area (Semba and Fibiger 1992; Paxinos and Watson 2007), concretely from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus.

The VTA receives as well inhibitory **GABA** inputs from:

- The ventral pallidum (VP) (Geisler and Zahm 2005)
- The NAc (Conrad and Pfaff 1976)

Other relevant inputs to the VTA are the **noradrenergic** from the locus coeruleus and the serotonergic from the dorsal raphe nucleus (Geisler and Zahm 2005; Phillipson 1976).

1.1.2 VTA OUTPUTS

Dopaminergic neurons in the VTA project to different central nervous system (CNS) targets. The projection to the NAc is the richest in dopaminergic neurons (65%–85%, e.g. 65-85% of the projections from the VTA to the NAc are dopaminergic), followed by those to the lateral septal area (72%), amygdala (53%), entorhinal cortex (46%), PFC (30%–40%), and hippocampus (6%–18%) (Fallon et al., 1984, Gasbarri et al., 1994, Margolis et al., 2006, Swanson 1982). It is also important to highlight that the electrophysiological and neurochemical properties of this DA neurons can differ based on their projection target (Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2006; Margolis et al., 2008).

In addition, a significant number of **GABA** and **Glu** neurons in the VTA project to the PFC and the NAc (Carr and Sesack 2000; Chuhma et al., 2004; Lavin et al.,2005; Margolis et al., 2006; Van Bockstaele and Pickel 1995).

Importantly, diverse studies using different retrograde markers have shown that each target receives input from a distinct subgroup of VTA neurons and rarely a subgroup of neurons sends projections to more than one single target (Fallon et al., 1984; Margolis et al., 2006; Swanson 1982). Therefore, there is growing evidence that not only the molecular phenotype of VTA neurons, but also their specific connectivity, determines the distinct part that these neurons play in reward and aversion (Brischoux et al., 2009; Morales and Margolis 2017; Salamone 2016).

1.2. OPIOIDERGIC CONTROL OF DA IN THE MCLS

The activity of DA neurons in the VTA is under the control, not only of the glutamatergic and GABAergic afferents aforementioned, but also of the **endogenous opioid system (EOS)**. The EOS has a critical role in mood regulation, reinforcement codification and also in the development of drug abuse (Gerrits et al., 2003). This system comprises three homologous G protein-coupled receptors (GPCRs) known as mu-, delta- and kappa- opioid receptors (MORs, DORs and KORs, respectively) and opioid receptor like-1 (ORL1), which are selectively activated by three groups of endogenous peptidic molecules: endorphins, enkephalins and dynorphins (Lord et al., 1977). Apart from their endogenous ligands, opioid receptors (ORs) can be also activated by several drugs. For example, morphine-like opiates have been demonstrated to bind preferentially to the MOR (Matthes et al., 1996).

It is now generally accepted that MORs are the site were **opiate drugs** exert their reinforcing properties (reviewed in Van Ree 1999, Le Merrer 2009). In fact, several studies using either pharmacological manipulations or knockout (KO) mice for the MOR have evidenced the critical role of this receptor in opiate addiction (Charbogne et al., 2014, Darcq and Kieffer 2018, Kieffer and Gavériaux-Ruff 2002). Very interestingly, the reinforcing properties of alcohol, cannabinoids, and nicotine are also strongly diminished in these MOR KO mice. A possible explanation for this involvement of MORs could be that those drugs would primarily activate their non-opiate targets, what would, in turn, trigger the release of endogenous opiates at appropriate MOR expressing sites in the brain. The genetic approach data also from MOR KO mice are less clear for cocaine and amphetamine, and it appears that MOR is not essential for the reinforcing properties of these drugs but it may play a modulatory role

(Charbogne et al., 2014). All these data underlie **MORs as mediators of drug reinforcement** by a direct (opiates) or indirect (non-opiates) activation.

Given this crucial role and the dense expression of MORs in the VTA (Bausch et al., 1995; Garzon and Pickel 2001; Sesack and Pickel 1992), numerous initial studies have been performed to evaluate the ability of **MORs to control DA neurons activity** and to mediate positive reinforcement. Different approaches have been used to address this issue.

For instance, **self-administration** paradigms have been traditionally used to study the reinforcing properties of drugs (for a deep review in behavioral paradigms used in the field see Sanchís-Segura and Spanagel 2006 and Belin-Rauscent et al., 2016). In these paradigms, animals usually learn to perform a task (e.g., press a lever) in order to receive an acute dose of a chosen drug. In a simple view, when the drug is able to increase DA activity, the animal will be reinforced to repeatedly perform the task (Di Chiara, 1998; Wise 2004). A wide number of studies using this paradigm have shown that animals intracranially self-administer MORs agonists into the VTA (reviewed in Wise 2004). Moreover, this behavior can be blocked by previously administering an antagonist for the MOR (Bozarth and Wise 1981).

Another widely used behavioral paradigm is the **conditioned place preference (CPP)**. In this case, animals receive the drug and they are immediately after confined in a specific context for repeated sessions. Thereafter, the preference of the animal for the drug-associated context in comparison to a neutral context is measured. The development of this drug-induced place preference is also considered a DA dependent behavior (Bozarth and Wise 1981; Spyraki et al., 1983; Wise 2004). Studies using this paradigm

have classically showed that both systemic and local microinjections of MOR agonists intra-VTA produce CPP (Hand et al., 1989; Shippenberg and Herz 1987). In the same manner as in self-administration studies, this intra-VTA CPP can be suppressed by a prior infusion of an antagonist of the MOR (Terashvili et al., 2004).

In addition, changes in **exploratory locomotion** in rats have been used as an indirect measure of DA neurons activation (Ikemoto 2010; Wise 2002). In general, drugs and natural reinforcers activate a common biological mechanism associated with approach behaviors and this, in the rodent model, results in the exploration of the environment and, therefore, in the increase in locomotion activity (Wise and Bozarth 1987). Numerous studies demonstrate that local administration of morphine into the VTA increases locomotor activity in rats (Bontempi and Sharp 1997; Devine and Wise 1994; Joyce and Iversen 1979).

A more direct analysis of DA activation can be performed by directly measuring **changes in DA levels** at dopaminergic terminal level in the MCLS. In this way, several studies also show an increase in DA release in the NAc after systemic or intra-VTA administration of MOR agonists (Devine et al., 1993; Gysling and Wang 1983). Finally, **electrophysiological** studies have also analyzed the effect of MOR agonists in DA activity. Ex vivo studies show that bath application of a MOR agonist activates putative VTA DA neurons (Johnson and North 1992; Matthews and German 1984). In accordance, systemic or intra-VTA morphine injection also increases putative DA neurons firing *in vivo* (Gysling and Wang 1983; Jalabert et al., 2011; Melis et al., 2000).

It is hence now clear that MOR agonism increases the activity of VTA DA neurons by activating local MORs. However, MORs are linked to $G_{i/o}$ family of G proteins and therefore their activation results in the **hyperpolarization of the MOR expressing neurons** (Williams et al., 2001). For that reason it was logical to assume that MORs are not located in DA cells, hence if so MORs agonism would reduce DA neurons activity. Early studies form Johnson and North 1992 aimed to study which neurons are directly responding to this MOR agonism. In this *in vitro* study, they showed that neurons that were directly hyperpolarized by MOR agonists were not identified as DA neurons. In turn, opioids induce the **hyperpolarization** of secondary (**GABA-containging**) **interneurons** in the VTA. Therefore, the direct action of opioids in these neurons results in a reduction of the spontaneous GABA-mediated synaptic input to the DA cells. Many electrophysiological studies both ex vivo and in vivo in anesthetized and freely behaving rats have further confirmed this observation (Steffensen et al., 2006; Xiao et al., 2007). In this way, MOR agonism in the VTA, acting through an indirect mechanism, would **disinhibit DA neurons** by removing the GABAergic tone.

Despite the aforementioned mechanism is nowadays generally accepted, alternative hypothesis have also been proposed to explain the action of local MOR agonism in the VTA. One of those suggests that the increase in DA release in the NAc induced by VTA MORs activation would occur via an indirect pathway. For example, some studies have shown that MOR agonists also inhibit the GABAergic neurons in the VTA that project to the NAc. The decrease in this GABAergic tone would, in second term, disinhibit the cholinergic neurons present in the NAc that receive this GABA afferents. Therefore, the **increase in acetylcholine would increase DA release in the NAc** through nicotinic acetylcholine receptors present on the striatal terminals

of DA neurons. (Cachope et al., 2012; Threlfell et al., 2012). Finally, there is also data suggesting that the synaptic action of MORs in the VTA is more diverse. In this way, a study carried out by Margolis and collaborators (Margolis et al., 2014) deeply explored the effect of MOR agonism across a large sample of neurons throughout the VTA. In this case, the authors evidenced an alternative mechanism by which **MORs would induce direct neuronal activation** through the opening of somatodendritic calcium channels. And, what is more intriguing, this mechanism was found in both DA and nonDA neurons. Moreover, this study, in contrast with the previous findings by Johnson and North 1992, also showed that MOR agonism induced both direct postsynaptic excitation and inhibition in VTA DA neurons.

All in all, it is of high importance to remember that, although the disinhibition model is the most commonly accepted, MOR effect on VTA neurons is a complex process and alternate mechanisms may contribute.

1.3 ETHANOL ACTION ON THE MCLS

Despite the fact that ethanol consumption represents an important contributor to the world burden of disease (Rehm et al., 2011; Rehm and Shield 2013; WHO 2018), the neurobiological mechanisms underlying its motivational properties still remain not fully discern. This fact is basically due to the particularity of ethanol physicochemical properties: it has a really simple chemical structure, with an ideal partition coefficient n-octane/water for crossing cellular membranes (including the blood-brain barrier) and also highly reactive. Given all those characteristics, the study of ethanol interactions with cellular elements as possible targets for its neurophysiological and behavioral effects has been a challenging task. Therefore, several hypothesis have emerged, not only implying ethanol direct action but also involving its metabolic products. In the following sections it can be found a brief compilation of the relevant aspects in this topic for the better understanding of the present work.

1.3.1 DOES ETHANOL ACTIVATE THE VTA DA NEURONS?

Ethanol is a **CNS depressant** that shares some behavioral effects with sedative-hypnotic drugs. Besides, ethanol is clearly **reinforcing and addictive** and these properties have been widely described both in humans and animals. Therefore, as other drugs of abuse, ethanol brain action results in the stimulation of DA neurons in the MCLS. In fact, intragastric (i.g.) (Enrico et al., 2009), intraperitoneal (i.p.) (Imperato and Di Chiara 1986) and intravenous (i.v.) (Howard et al., 2008) ethanol administration enhances DA transmission in the NAc. Moreover, several researches point to the fact that ethanol exerts its action in the VTA. In this line, the administration of ethanol directly into the VTA triggers an increase in the DA release into the NAc (Di

Chiara and Imperato 1988; Gonzales et al., 2004; Howard et al., 2008; Imperato and Di Chiara 1986). There are also a wide number of studies showing that this local action of ethanol induces excitation of VTA DA neurons *in vitro* (Brodie et al., 1990; Brodie et al., 1999b; Melis et al., 2009; Xiao et al., 2007), as well as *in vivo* (Foddai et al., 2004; Gessa et al., 1985; Mereu and Gessa 1984). However, the exact mechanism through which ethanol exerts this DA activation is still not clear and so far different hypothesis have been postulated. Among those, it has been reported that ethanol interacts with different neurochemical and endocrine systems of the CNS such as the GABAercgic, glutamatergic, dopaminergic, opioidergic, cannabinoid or corticotropin releasing factor (CRF). In addition, ethanol can also act on different ion channels (calcium, potassium) and modulate cytoplasmatic components such as second messengers (Erdozain and Callado 2014; Morikawa and Morrisett 2010). Not only it is not yet clear which of those is the mechanism that is activating DA neurons, but also it is in doubt if it constitutes a direct or indirect process.

Regarding ethanol **direct action**, electrophysiological studies have shown that local application of ethanol results in the activation of VTA DA neurons by acting on the ionic channels that modulate their activity. Concretely, it **increases the Ih current** (mixed Na+/K+ conductance) and **reduces the voltage-gated transient K+ current** (Brodie et al., 1990; Brodie and Appel 1998; Brodie et al., 1999a; 1999b; Koyama et al., 2007; Okamoto et al., 2006). It should be taken into account, that increases in Ih current result in neuronal activation, whereas increases in voltage transient K+ current result in neuronal inhibition. Therefore, the previously mentioned ethanol direct action on those two mechanisms would result in an increase in DA neuron firing.

Apart from its direct action, it has been suggested that other **indirect mechanisms** should be contributing to the ethanol-induced activation of DA neurons. This idea is based on the differences found between *in vitro* and *in vivo* electrophysiological studies, such as the magnitude of the effect of ethanol on DA neurons (that appears to be larger *in vivo* (Gessa et al., 1985; Mereu and Gessa 1984; Stobbs et al., 2004) than *in vitro* (Brodie et al., 1990; Brodie et al., 1999b; Brodie and Appel 1998; Brodie and Appel 2000; Okamoto et al., 2006)) or the different sensitivity to ethanol between VTA and SN DA neurons that is manifested *in vivo* (Gessa et al., 1985) but not *in vitro* (Okamoto et al., 2006). Thus, ethanol has been proposed to interact with the different afferents that innervate the VTA DA neurons, mainly the glutamatergic and GABAergic (Lovinger 1997; Morikawa and Morrisett 2010; Vengeliene et al., 2008).

In the case of glutamatergic afferents, it has been reported that acute ethanol exposure can indirectly **enhance glutamatergic transmission onto VTA DA neurons**. The proposed mechanism is based on the fact that systemic or local brain administration of ethanol increases DA levels in the VTA (Campbel et al., 1996; Kohl et al., 1998; Yan et al., 1996). This somatodendritic release of DA activates presynaptic DA type 1 (D_1) receptors located in the glutamatergic afferents in the VTA and consequently increases glutamate release (Deng et al., 2009, Xiao et al., 2009). Then, the activation of ionotropic glutamate receptors in DA neurons would stimulate VTA DA neuron firing (Christoffersen and Meltzer 1995, Zhang et al., 1997).

On the other hand, VTA GABA neurons have been also implicated as a possible mediator of ethanol action on VTA DA firing. In fact, it has been shown that **ethanol can inhibit GABA neurons** (Gallegos et al., 1999; Steffensen et al., 2000; 2009; Stobbs et al., 2004) and, consequently, **disinhibit**

DA neurons (see section 1.2) (Johnson and North 1992; Mereu and Gessa 1985; Xiao et al., 2007). As commented in section 1.2, the activity of GABA neurons is controlled by MORs and MOR agonism inhibits these neurons, resulting in the activation of DA neurons. According to this, some authors have demonstrated that ethanol action on DA activity is **mediated by MORs**. Both *in vivo* and *in vitro* electrophysiological studies show that ethanol alters the GABAergic transmission onto VTA DA neurons, and this action requires MORs (Guan and Ye 2010; Xiao et al., 2007; Xiao and Ye 2008). Moreover, behavioral studies have also reported that ethanol-induced DA-dependent behaviors are decreased or prevented by the blockade of local MORs in the VTA (Gajbhiye et al., 2017; Gibula-Bruzda et al., 2015; Quintanilla et al., 2014; Sánchez-Catalán et al., 2009). Finally, there is also a large body of literature demonstrating that the blockade of the opioidergic system by antagonists of the ORs is highly correlated with the reduction in ethanol consumption in rodents and also in humans (Samson and Doyle 1985; Hubbell et al., 1986; Reid et al., 1991; O'Malley et al., 1992; Volpicelli et al., 1992).

1.3.2 HOW DOES ETHANOL ACTIVATE VTA MORs? ROLE OF ETHANOL METABOLITES

As previously mentioned, MORs are involved in ethanol effects, and this interplay has been widely explored for several decades (see Nutt 2014 for review). Given that the ethanol molecule *per se* is not considered an opioid-like agonist, there should be an indirect mechanism that ultimately activates MORs after ethanol administration. Previous studies have proposed that the **balance between the presence of ethanol molecule and its metabolic products** in the MCLS would determinate these final activating and reinforcing ethanol properties (metabolites hypothesis).

According to the metabolites hypothesis, enzymatic systems responsible for the biotransformation of ethanol in the brain, play a crucial role in ethanol regulation of VTA DA neurons activity. The ethanol molecule is able to easily cross the blood-brain barrier and be metabolized in the brain to **acetaldehyde (ACD)**, its first metabolite (Cohen et al., 1980; Raskin and Sokoloff 1970). ACD is a highly reactive compound that can easily condensate with diverse endogenous molecules, such as biogenic amines, to produce different biological active compounds, including salsolinol (SAL) (derived from the condensation of ACD and DA) (Cohen and Collins 1970; Cohen 1976; David and Walsh 1970; Walsh et al., 1970). Based on this fact, it is possible that the activation of VTA DA neurons observed after ethanol administration is, in turn, elicited by its metabolic products in the brain. In fact, it is difficult to discern wether the aforementioned described events following ethanol administration in the VTA (section 1.3.1) derive from a direct action of the ethanol molecule or from the action of ethanol metabolites. In this last possibility, depending on the administered dose and, therefore, on the relative saturation of the enzymes and the balance in the ratio ethanol/metabolites concentration, we will observe

activation (Figure 1.3), inhibition (Figure 1.4) or, even, no effect on the activity of the DA neurons of the MCLS. Following, a schematic representation of the ethanol dual action on VTA DA neurons is presented in Figure 1.3 and Figure 1.4 for the better understanding of the metabolites hypothesis:

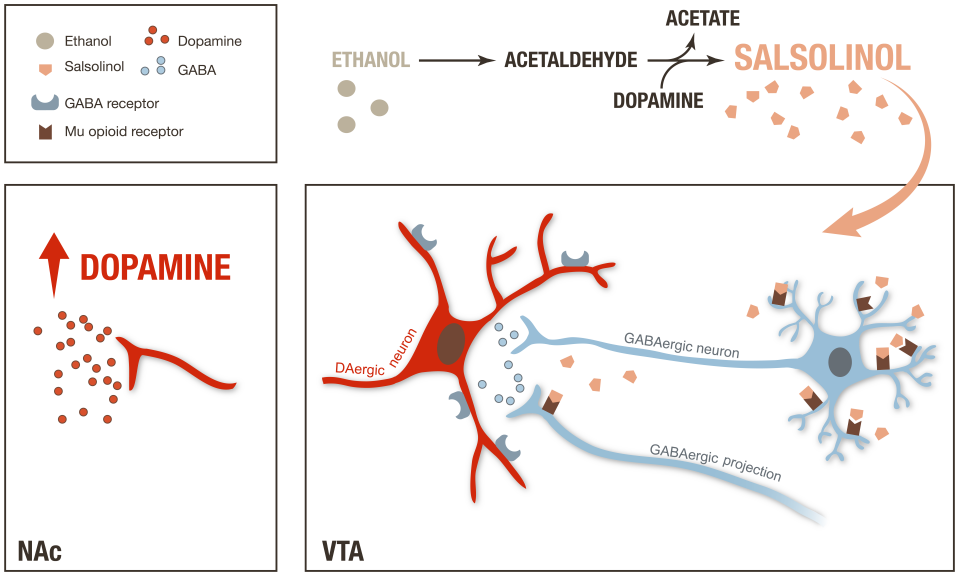

Figure 1.3 Activating effect of ethanol metabolized fraction on the VTA DA neurons. According to the aforementioned metabolites hypothesis, ethanol reinforcing properties would be the result of ethanol dual action over the MCLS DA neurons. Thus, ethanol metabolite, salsolinol, is the responsible for the DA neurons activation. This excitation seems a consequence of the reduction of GABA release derived from the activation of the mu-opioid receptors by salsolinol. Abbreviations: NAc, nucleus accumbens; VTA, ventral tegmental area.

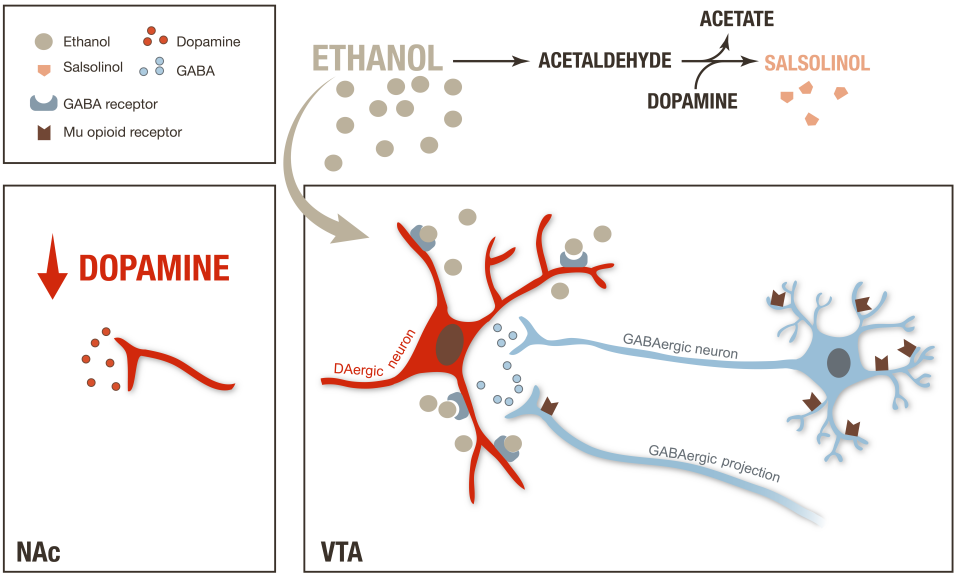

Figure 1.4 Inhibitory effect of ethanol non-metabolized fraction on the VTA DA neurons. According to the aforementioned metabolites hypothesis, ethanol reinforcing properties would be the result of ethanol dual action over the MCLS DA neurons. Thus, the ethanol molecule itself (non-metabolized fraction), acting on the $GABA_A$ receptors, promotes the enhancement of GABA release, and consequently, the inhibition of DA neurons. Abbreviations: NAc, nucleus accumbens; VTA, ventral tegmental area.

The metabolites hypothesis has been confirmed by previous studies in our group by Martí-Prats and collaborators (Martí-Prats et al., 2013; Martí-Prats et al., 2015). In those studies, changes in exploratory locomotion in rats were measured, as this phenomenon is considered the behavioral reflection of the activation of VTA DA neurons (Wise 2002). In a very elegant approach, the authors selected an inactive ethanol dose that did not produce changes in locomotor activity when administered into the VTA. Thereafter, different pharmacological strategies were designed to preferentially test either the effects of the metabolized or non-metabolized ethanol fraction (see Figure 1.3 and Figure 1.4): (i) To test the non-ethanol metabolized fraction effect, the selected pharmacological approach was to decrease the presence of the ethanol

metabolites, either by inhibiting the enzyme responsible for the ACD formation, or by sequestering the ACD with D-penicillamine. In this case, this ethanol inactive dose produced a decrease in the rats locomotor activity (Martí-Prats et al., 2013). Moreover, the same effect was found when blocking MORs, further supporting the role of these receptors on the activating effects of ethanol (Martí-Prats et al., 2015). And (ii), to identify the metabolized fraction effect, the selected strategy was, in this case, to increase ethanol metabolites *in situ* production by inhibiting ACD degradation. This manipulation converted the inactive ethanol dose into a stimulating one.

All this data point to **ethanol-metabolized fraction** as the responsible for the **activating effect of ethanol on VTA DA neurons**. One of the ethanol metabolites that appear to be the latest responsible for this effect is **SAL** (Hipólito et al., 2012), the product of the condensation of ACD and DA (Haber et al., 1996; Melis et al., 2015). Several findings support the idea that ethanol-derived **SAL activates MORs in the VTA** and, therefore, disinhibits VTA DA neurons (see Figure 1.3). First, SAL presents structural similarities with isoquinoline alkaloids (such as morphine) (Davis and Walsh 1970). Second, SAL can bind to ORs and produces opiate-like effects (Berríos-Cárcamo et al., 2016; Fertel et al., 1980; Lucchi et al., 1982). And, third, SAL is able to increase both NAc DA extracellular levels and the firing rate of DA neurons, effects that are mediated through MOR activation (Hipólito et al., 2010; Xie et al., 2012). Finally, there is also electrophysiological evidence supporting the key role of SAL in ethanol action on the VTA (Melis et al., 2015). In this study, the *in situ* formation of SAL, derived from the local application of ethanol or ACD in VTA slices, was impaired by removing DA content, a chemical substrate of this reaction. As a result, when restricting SAL formation, neither ethanol nor ACD aimed to stimulate VTA DA neurons.

Although these gathered evidences point to SAL to exert the excitatory effects of ethanol on VTA DA neurons, we are still lacking the direct confirmation of the SAL formation in the VTA that would definitively confirm this hypothesis.

On the other hand and according to the aforementioned study from Martí-Prats and collaborators, ethanol (more specifically, the **non-metabolized fraction of ethanol**) would, by its own, exert a **depressant effect** on animal motor behavior (Martí-Prats et al., 2013). This depressant effect has been proposed to be the result of ethanol direct action on GABA neurons (see Figure 1.4) (Xiao et al., 2009). Actually, the inhibition of $GABA_A$-Rs by the administration of the antagonist bicuculline was able to convert the previously inactive ethanol dose into an activating one (Martí-Prats et al., 2013). This phenomenon has been evidenced not only by this behavioral approach but also electrophysiological studies have endorsed this ethanol depressant action (Theile et al., 2011, 2009, 2008).

Despite the evidence described above, there are **alternative hypothesis** to explain the ethanol derived activation of MORs in the VTA. The most extended is the theory that points to the endogenous peptide **β-endorphin** as the main responsible for this action (Sanchís-Segura et al., 2005; Xiao et al., 2007; Xiao and Ye 2008). This hypothesis is based on the fact that systemic ethanol administration induces the release of this endogenous peptide in the VTA (Rasmussen et al., 1998; Jarjour et al., 2009). Moreover, local application of ethanol in slices from the hypothalamic arcuate nucleus (a region that sends projections to the VTA) increases β-endorphin synthesis (Gianoulakis 1990). In addition, it has been reported that the ability of ethanol to stimulate β-endorphin release is mediated by ethanol metabolized fraction in the hypothalamic arcuate nucleus and that ACD exposure in this region also

promotes β-endorphin release even more potently than ethanol (Reddy and Sarkar 1993, Sanchís-Segura et al., 2005). However, it should be pointed that there is **no direct evidence** that the administration of ethanol or its derivatives into the VTA elicits the local release of β-endorphin, so this theory might not be able yet to explain all the behavioral and electrophysiological results described above derived from intra-VTA ethanol administrations.

1.3.3 ETHANOL-INDUCED ADAPTATIONS IN THE MCLS

As previously mentioned, ethanol, like other drugs, activates the DA neurons in the MCLS. One of the consequences of the continuous activation of the DA neurons is the development of **glutamatergic neuroadaptative** responses in the NAc at pre- and post-synaptic level (reviewed in Hearing et al., 2018) but also in other brain areas like for example the hippocampus (Fakira et al., 2014; Portugal et al., 2014). These adaptations in synaptic function play a crucial **role in associations between the drug and the drug administration environment** and can lead to cravings that promote continued use of the drug and can facilitate relapse (Daglish et al., 2001; See 2002).

Glutamate receptors comprise two large families, ligand-gated ion channels (ionotropic receptors) and GPCR (metabotropic receptors). Ionotropic receptors are, in turn, divided into three classes: **α**-amino-3-hydroxi-5-methyl-4-isoxazole propionic acid receptors (AMPARs), kainate receptor and N-methlyd-D-aspartate receptors (NMDARs). Concretely, **NMDARs play an important role in synaptic plasticity**. These receptors show an important biophysical property: they allow calcium entry only if the cell is depolarized. This means that NMDARs act as synaptic coincidence

detectors to facilitate plasticity. Postsynaptic (or presynaptic) Ca^{2+} influx through NMDARs, in turn, activates intracellular singling cascades that ultimately are responsible for changes in synaptic efficiency. NMDARs are heterotetramers mainly composed by two subunit classes: two **NR1 subunits** and two **NR2 subunits**. (Lüscher and Malenka 2012). The subtype of NR2 subunit (NR2A-D) can influence the biophysical and pharmacological properties of endogenous NMDARs (Cull-Candy et al., 2001; Monyer et al., 1994). From those, NR2A and NR2B are the most common NR2 subunits in the adult forebrain. NR2B is the most common also in the early postnatal forebrain and it is replaced to some extent by NR2A during development (Aizeman and Cline 2007; Cline et al., 1996; Tovar and Westbrook 1999). Within all neuronal types, NMDAR complexes are found at the synapse and in extra synaptic locations, with the later made up mainly of NR1-NR2B and NR1-NR2D (Gereau and Swanson 2008).

Local **NMDARs** in the **NAc** play an important role in the **development of context-learned associations** driven by both natural rewards (Brigman et al., 2013; Dang et al., 2006; Parker et al., 2011; Yin et al., 2008) and drugs of abuse (Beutler et al., 2011; Hearing et al., 2017; Heusner and Palmiter 2005). It is now widely accepted that this region is fundamental in drug-seeking behaviors, including alcohol (Chaudhri et al., 2008, Fuchs et al., 2008). In the NAc, synaptic plasticity mechanisms take place in the glutamatergic synapses. These glutamatergic afferents arise from limbic areas such as prefrontal cortex, hippocampus and BLA (Brog et al., 1993, Sesack and Grace 2010). The excitatory input acts onto the **GABAergic medium-spiny neurons** (**MSNs**), that constitute the majority (~95%) of all neurons in the NAc. Given the importance of NAc glutamatergic changes for drug-induced learned associations, several studies have deeply analyzed the involvement of local

NMDAR subunits in these processes. Unfortunately, not many of those studies have focused their attention on ethanol-induced changes in accumbal NMDAR. Concretely, a study by Sikora and colleagues shows that the NR1 subunit is necessary for the development of associations between context and ethanol (and other drugs of abuse) (Siroka et al., 2016). This study uses a genetic strategy to selectively inactivate the NR1 subunit in neurons expressing D_1 receptors. Therefore, in the NAc of the mutant mice, only neurons that are activated by DA (D_1 containing) did not expressed the NR1 subunit. And, hence, those mutant mice did not develop a preference for a context associated with the administration of ethanol or other drugs. Moreover, the local administration of a non-specific NMDAR antagonist (AP-5) intra-accumbens also blocks ethanol-induced CPP (Gremel et al., 2009). From those studies it appears that accumbal NMDARs play an important role in associations between ethanol and specific environments, what may have critical consequences in seeking behaviors. Therefore, it is of high relevance to further explore how these receptors modulate context-learned associations induced by ethanol and which is the specific role of the different NMDAR subunits. Finally, **chronic ethanol exposure** has also been shown to induce NAc glutamatergic adaptations. In ethanol-dependent rats, NAc neurons show an increase in NMDAR sensitivity (Siggins et al., 2003). In the same way, glutamatergic transmission onto D_1 MSN has been reported to be enhanced after chronic ethanol exposure (Renteria et al., 2017). Moreover, withdrawal from chronic ethanol increases the expression of NR1 and NR2B subunits in the NAc (Zorumski et al., 2014).

On the other hand, there is also great evidence that repeated drug treatment induces synaptic plasticity in the **hippocampus** and that those changes are involved in drug-context associations. Plenty of work supports the

fact that several forms of **hippocampal synaptic plasticity**, such as long-term potentiation (LTP), are mediated by **NMDAR** signalling (Muller et al., 1988; Shapiro and Eichenbaum 1999; Miyamoto 2006). Therefore, hippocampal changes in NMDARs may be crucial for **contextual driven memories**, what finally implies consequences in drug dependence and craving (Daglish et al., 2001; Ersche et al., 2006; Prosser et al., 2006). However, as it has been previously commented for the NAc, very few studies have explored this role of hippocampal NMDAR in ethanol-derived context association. Given that ethanol action is mediated by ORs, it would be logical to assume that opioid-induced glutamatergic changes in the hippocampus could also take place in the case of ethanol. Expression of opiate-induced CPP has been associated with increased basal hippocampal synaptic transmission, impaired hippocampal LTP, and increased synaptic expression of the NR1 and NR2B subunits in the hippocampus (Portugal et al., 2014). Unfortunately, wether those adaptations are also elicited in ethanol-induced context associations remains unknown. In a similar manner as in the NAc (see above), it has been reported that **chronic ethanol exposure** also induces changes in hippocampal NMDAR expression. In particular, ethanol chronic ingestion up-regulates NR1 receptor subunit expression in the hippocampus (Kalluri et al., 1998; Spencer et al., 2016; Trevisan et al., 1994). Additionally, some studies have explored changes in NR2 subunits after repeated ethanol exposure and they have reported an increase in hippocampal NR2A and NR2B subunits expression (Carpenter-Hyland et al., 2004; Chandler et al., 2006; Hendricson et al., 2007; Kalluri et al., 1998; Snell et al., 1996; Spencer et al., 2016). However, it is still not clear if those changes may differ depending on timing and/or administration pattern differences.

1.3 ALCOHOL USE DISORDER AND PAIN

Chronic pain affects millions of people in the world and chronic pain patients usually suffer comorbid emotional disorders (e.g., anxiety, depression) and cognitive deficits (e.g., memory impairment) (Bair et al., 2003; Bushnell et al., 2013; Fine 2011). Also the presence of pain has been reported to alter drug consumption, probably **increasing the vulnerability of pain patients to suffer substance use disorders** (SUDs) (Rosenblum et al., 2003; Potter et al., 2008). In fact, the interplay between pain and opioid abuse is nowadays profoundly studied due to the alarming data reporting an emerging opioid crisis, specially in the USA (Elman and Borsook 2016; Volkow and McLellan 2016). Moreover, clinical data have shown a positive correlation between persistent **pain with an increased risk of developing AUD** (Witkiewitz et al., 2015a). Therefore, this section gives first a general view of the AUD problematic, focusing on relapse. Following, it provides a brief description of pain effect on drugs motivational action on the MCLS and, finally the interaction between pain and AUD is analyzed.

1.3.1 ALCOHOL USE DISORDER: A RELAPSING DISEASE

SUDs are chronic recurring diseases with a multifactorial etiology including genetic, neurobiological, psychological and environmental factors (Jupp and Lawrence 2010; Koob and Le Moal 2006). According to the Diagnostic and Statistical Manual of Mental Disorders (DSM, 5th edition), AUD is a problematic pattern of alcohol use leading to clinically significant impairment or distress. In Europe, AUD is highly prevalent (World Health Organization, WHO, regional office for Europe) and very resistant to pharmacological treatment (for detailed review see Litten et al., 2012).

Data from latest EDADES study in **Spain** (2017), represented in Figure 1.5, shows that the prevalence of alcohol consumption remains steady and greater to other drugs (75% of the population has consumed alcohol in the last year) and it sets the high-risk consumers prevalence in 5.7%. Also data form the WHO reveals that total amount of alcohol consumption per capita has not changed during the last 10 years (10 liters/year) and that data in Spain are equivalent to the average in Europe (Global status report on alcohol and health 2018, WHO). Finally, it has been reported that approximately 200,000 of the population in Spain suffer from AUD (1.2% of men and 0.2% of women) (Rehm et al., 2012). These data are equivalent to other treatable chronic medical conditions such as schizophrenia (0.3-0.8%) (Ayuso-Mateos et al., 2006).

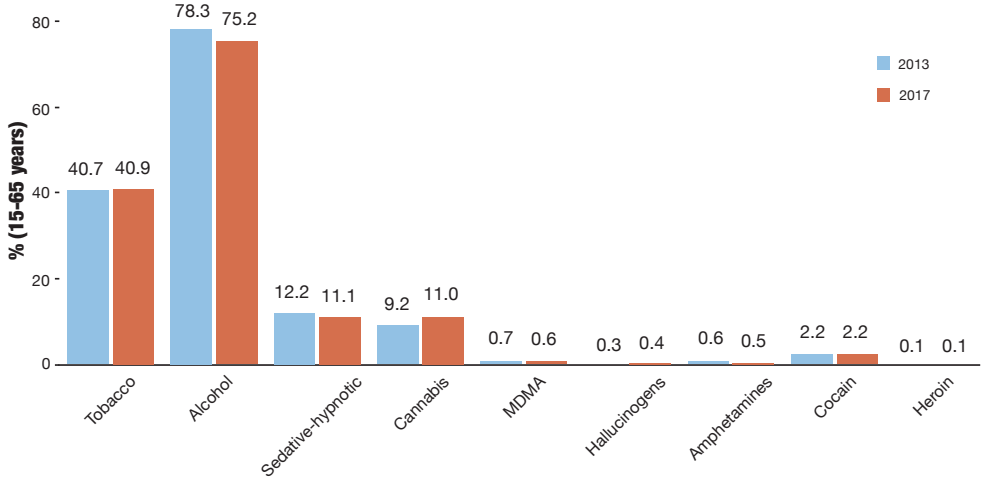

Figure 1.5. Percentage of drug users (15-65 years) in Spain. Graphical representation adapted from the alcohol and drug survey in Spain by "Plan Nacional sobre Drogas" (Ministerio de Sanidad, Servicios Sociales e Igualdad 2017).

Moreover, alcohol consumption not only constitutes a health problem by itself, but it has also been identified as an important **risk factor for chronic disease and injury** (Lim et al., 2012; Rehm et al., 2009). Data from the Global status report on alcohol and health 2018 (WHO) shows that the harmful use of alcohol resulted in an estimated 3 million deaths (5.3% of all deaths) globally in 2016. The leading contributors to those alcohol-attributable deaths were digestive diseases, unintentional injuries and cardiovascular diseases, being individually responsible for 21.3%, 20.9% and 19.0% of these deaths, respectively (Global status report on alcohol and health 2018, WHO). Finally, the costs associated with alcohol amount to more than 1% of the gross national product in high-income and middle-income countries, with the costs of social harm constituting a major proportion in addition to health costs (Rehm et al., 2009).

1.3.1.a Alcohol relapse is a crucial factor for AUD treatment

Plenty of researches have described SUDs as chronic relapsing disorders characterized by alternating cycles of remission and relapse (Gossop et al., 2003; Hser et al., 1993; White et al., 2002). Alcohol and, in general, drugs of abuse, induce changes in gene expression regulation, molecular alterations, and synaptic and cellular changes, being some of them persistent even after detoxification (Noori et al., 2012, Spanagel et al., 2013). Those alterations are, in part, responsible for a permanent risk of relapse in alcohol-dependent individuals. In fact, **preventing relapse** during long abstinence periods is nowadays the **main clinical challenge** that undergoes the therapeutical strategies **for treating alcohol-dependent patients after detoxification** (Johnson 2008).

According to the WHO recommendations, prevention of relapse may involve psychosocial (a combination of psychological and social) and pharmacological interventions. Pharmacological interventions used in alcohol dependence for prevention of relapse include acamprosate, disulfiram, cyanamide, naltrexone and nalmefene (this last only in Europe). However, recent data reveal that there is still low efficacy of current interventions, hence at most 50% of treated people do not achieve remission after long follow up periods (Fleury et al., 2016).

As previously mentioned, **AUD** undergoes with several **neuroadaptations** derived from the excessive engagement of the reward system. The **EOS** plays an important role among those, being, therefore, the target for some of the existing pharmacological treatment for relapse and for the possible new ones still under preclinical and clinical investigations. Concretely, continued regular alcohol use can lead to (i) a decreased reward

function and (ii) an increased activation of brain stress systems, which motivates compulsive alcohol intake (Koob 2014). Regarding the first, several data confirmed a dysregulation of DA and EOS during abstinence both in alcohol dependent humans and rats (for a recent review see Hansson et al., 2019). Precisely, a **downregulation of MORs can be found during abstinence** (Hermann et al., 2017; Hirth et al., 2016; Laukkanen et al., 2015; Oliva and Manzanares 2007). In fact, naltrexone and nalmefene produce their anti-relapse effects through the MOR (Heilig et al., 2011; Palpacuer et al., 2015), although it is still not clear why they do not have a high impact in the clinical practice (Jonas et al., 2014; Palpacuer et al., 2018). Parallel to the decreased function of the reward system is the increase in stress related behaviors and negative affect, in which KORs and their endogenous ligand dynorphin play a critical role (Bruchas et al., 2010; Koob 2014). **Prolonged alcohol exposure** is related with an **increased KOR sensitivity** that facilitates negative affective states (Heilig et al., 2010; Meinhardt and Sommer 2015), as well as escalated alcohol consumption (Berger et al., 2013; Kissler et al., 2014; Walker and Koob 2008). For that, kappa antagonists also appear to be good candidates for pharmacologically prevention of alcohol relapse and they have shown to be effective in a preclinical setting (Domi et al., 2018; Harshberger et al., 2016; Walker et al., 2011). Unfortunately, the clinical development of this pharmacological strategy has not been achieved yet.

After all, there is a general agreement about the necessity of deeper understanding the neurological mechanisms that mediate relapsing behaviors and the risk factors that could increase the vulnerability to relapse. A better insight of this phenomenon would, therefore, lead to the development of new pharmacological treatments that, together with psychotherapeutic interventions, would reduce the rate of alcohol relapse prevention.

1.3.1.b The ADE preclinical model for studying relapse behavior

Nowadays, preclinical studies with appropriate animal models are still needed to investigate the neurobiology of relapse behaviors. These models are, obviously, not able to fully reproduce the complexity of the entire spectrum of AUD. However, decades of research support the hypothesis that animal models constitute an excellent platform for the development on new pharmacological treatments (Belin-Rauscent et al., 2016; Bell et al., 2012; Bossert et al., 2013; Ciccocioppo 2013; Vengeliene et al., 2008).

Regarding the study of relapse, the **Alcohol Deprivation Effect (ADE)** is one of the most frequently explored relapse-related behaviors. Sinclair and Senter first reported in 1968 that rats exposed to long periods of voluntary access to ethanol, showed a **robust but transitory increase in drug intake after a forced abstinence period**, a phenomenon referred to as ADE (Sinclair and Senter 1968). Since then, the ADE has been so far described in rats (Herz 1997; McKinzie et al., 2000; Rodd-Henricks et al., 2000; Sinclair and Li 1989; Spanagel and Holter 1999), but also in mice (Salimov and Salimova 1993), monkeys (Kornet et al., 1991) and even humans (Burish et al., 1981). However, in rats and mice, the occurrence and magnitude, as well as the duration, of the ADE are strongly dependent on the genetic background and thus a huge variability among strains is observed (Rosenwasser et al., 2013, Vengeliene et al., 2003). Moreover, this phenomenon has been observed both in operant and non-operant self-administration paradigms (Martin-Fardon and Weiss 2013; Martí-Prats et al., 2015; Orrico et al., 2013). All this data further support that the ADE has a strong construct validity to be used in alcohol-relapse research. In addition, current treatments used to treat AUD, such as acamprosate and naltrexone, are able to reduce or impair the ADE (Sinclair and Sheaff 1973; Spanagel and Holter 1999; Spanagel and Kiefer 2008). These

pharmacological confirmation provides prediction validity to this model for the development of new treatments.

Another characteristic of this model is that, together with the increase of ethanol intake, the ADE has been reported to be resistant to manipulations of ethanol concentration, taste, and environmental factors (Spanagel et al., 1996; Wolffgramm and Heyne 1995; Vengeliene et al., 2009). This fact supports the idea that after an abstinence period, animals display a compulsive drinking behavior, which constitutes one of the main diagnostic criteria for AUD. This has been demonstrated by showing a resistance of the ADE to modification by changes in the palatability of ethanol via quinine or sucrose addition, or by manipulation of environmental and social conditions (Spanagel et al., 1996; Vengeliene et al., 2009).

For all that, several recent studies in rodents have selected the ADE for studying the neurobiological mechanisms that mediate alcohol relapse as ell as a preclinical model for the development of new anti-relapse pharmacological treatments (Fredriksson et al., 2019; Gajbhiye et al., 2018; Quintanilla et al., 2019; Martí-Prats et al., 2015a; Orrico et al., 2013; Söderpalm et al., 2019; Uhari-Väänänen et al., 2019; Zhou and Kreek 2019).

1.3.2 PAIN AND THE MCLS

The International Association for the Study of Pain (IASP) defines pain as "**an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage**" (http://www.iasp-pain.org). The aversive emotions associated with pain are essential for its adaptive role. In healthy subjects, these alerting and aversive components trigger escape from danger and avoidance of future harm. When it becomes chronic, however, pain loses its survival value and becomes a medical problem. The **MCLS** plays an important **role in the emotional aspects of pain** and it has been reported that **persistent pain can induce changes** in this system. Therefore, this section starts by providing a general view of pain processing mechanisms and then describes the impact of pain on both the EOS and the motivational and reward processing (for a complete review see Taylor 2017).

1.3.2.a Central mechanisms of pain

The **ascending nociceptive pathway** begins with peripheral primary nociceptors. Primary nociceptors are bipolar neurons with one of their axonal branches innervating peripheral tissues (skin, muscles, joints, viscera). The primary nociceptors that innervate the body have their cell body located in the dorsal root ganglia (DRG), whereas the ones that innervate the face have their cell body located in the trigeminal ganglion. As specialized primary afferents, they are activated by noxious stimuli and transduce them into action potentials. Then, they conduct the generated action potential to the dorsal horn of the spinal cord through their second axonal branch. The nociceptive information is carried to the brainstem (spinoreticulothalamic tract) and to the thalamus (spinothalamic tract), constituting the ascending pathway. From these brainstem and thalamic loci, information reaches cortical structures (Fields

2004; Nestler et al., 2009). A simplified representation of pain ascending pathways can be found in Figure 1.6. Many brain areas are activated by pain, being some of them generally associated with the sensory-discriminative properties (such as the somatosensory cortex) and others with the emotional aspects (such as the anterior cingulate gyrus and insular cortex) (Basbaum et al., 2009; Willis and Coggeshall 1991).

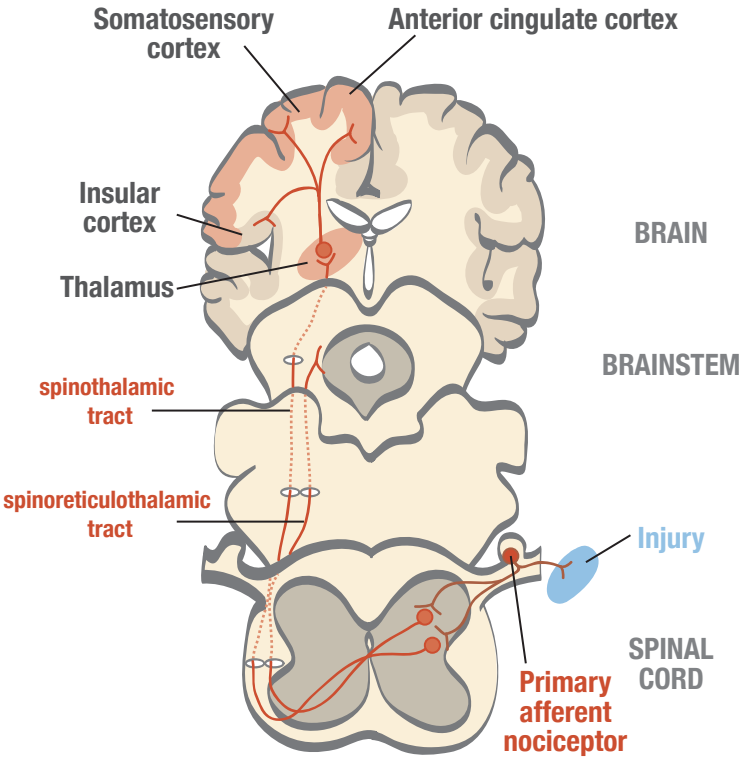

Figure 1.6. Schematic representation of afferent pathways underlying the sensation of pain. Injury activates the primary afferent nociceptor, which transmits information to the dorsal horn of the spinal cord and activates the second order neurons. The axons of nociceptive dorsal horn neurons cross to the contralateral anterolateral quadrant to form an ascending tract, which terminates in the brainstem and several distinct areas of the thalamus. The thalamus contains higher order neurons that project to various cortical regions that mediate different aspects of the pain experience. These regions include somatosensory, anterior cingulate and insular cortices. (Adapted from Fields 2004)

Pain-associated cortical areas send **descending axons** to many brainstem regions that ultimately terminate in the spinal and trigeminal dorsal horn. This descending projections act as a **modulatory system** that regulates the output from the spinal cord, thereby **inhibiting or amplifying the flow of painful stimuli.** Initial work from Reynolds 1969 followed by Liebeskind and colleagues (Mayer et al., 1971; Mayer and Liebeskind 1974; Reynolds 1969) pointed to a specific region of the midbrain — the periaqueductal grey **(PAG) as a key area in these top-down processes** (reviewed in Baskin et al., 1986; Hosobuchi et al., 1977). The PAG receives direct inputs from the hypothalamus and from the limbic forebrain, including several regions of the frontal neocortex and the CeA. Afferents from the PAG arrive to the rostral ventromedial medulla (RVM) and the dorsolateral pontine tegmentum (DLPT), that, in turn, project to the spinal cord. A simplified representation of this descending pathway can be found in Figure 1.7. According to this circuitry, the PAG can indirectly control pain transmission by inhibiting or enhancing the activity of dorsal horn neurons that respond to noxious stimulation (Fields 2004). In addition, the **RMV also plays an important role in pain regulation**. Curiously, the activation of RMV neurons can both facilitate or inhibit pain transmission (Porreca et al., 2002; Urban and Gebhart 1999). This mechanism is derived from the activity of two neuronal subpopulations: "on cells" and "off cells", both present in the RMV that, respectively, facilitate or inhibit nociceptive transmission at the level of the dorsal horn (Fields et al., 1995; Fields 2004).

It is now widely accepted that analgesic action of opioids is mediated by their action on this circuitry. At spinal level, MOR agonists can act on primary afferents (Stein et al., 2003) or nociceptive neurons in the spinal cord (Glaum, et al., 1994; Grudt and Williams 1994) and, therefore, directly inhibit pain

transmission. On the other hand, **MORs are present in all the aforementioned supraspinal components of the pain-modulation** circuit including the insular cortex, amygdala, hypothalamus, PAG, DLPT, RVM and spinal cord dorsal horn (Akil et al., 1998; Arvidsson et al., 1995; Mansour et al., 1988; Mansour et al., 1995). In fact, local microinjection of MOR agonists administered into all those regions has been shown to inhibit noxious stimulation (Burkey et al., 1996; Fields et al., 1991; Manning et al., 1994; Yaksh and Rudy 1978). In addition, endogenous opioid release is also involved in this pain-modulation mechanism. Indeed, the microinjection of an opioid antagonist at a downstream site in the pathway is able to block the effect produced by the local activation at a different level (Kiefel et al., 1993; Roychowdhury and Fields 1996; Tershner and Helmstetter 2000).

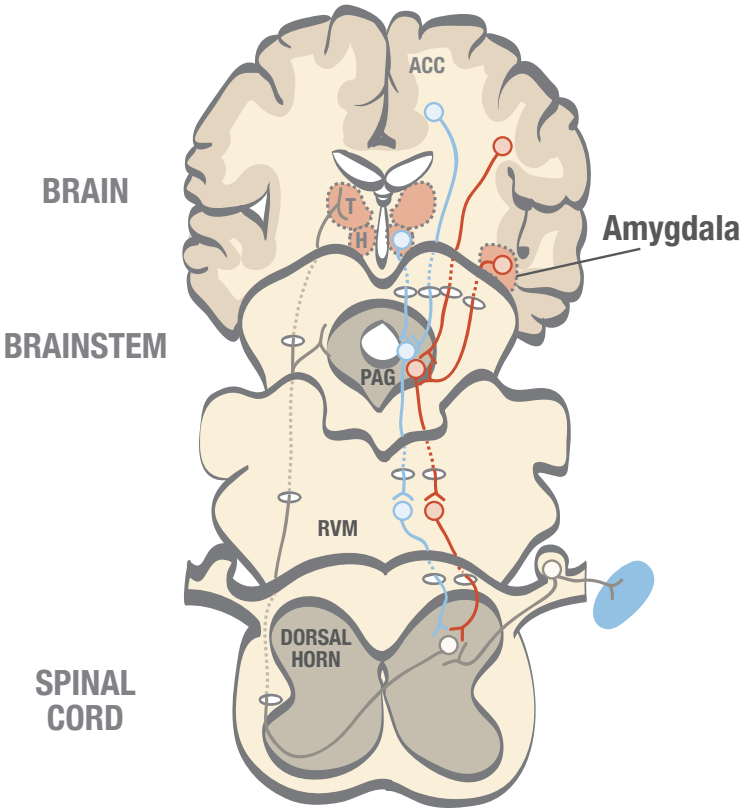

Figure 1.7. Outline of opioid-sensitive pain-modulating circuit. This constitutes a top–down pathway that can be activated by both exteroceptive stimuli and certain motivational states. Limbic forebrain areas, including the ACC, other frontal cortical areas, the H and the amygdala project to the midbrain PAG, which can be considered as a main output pathway of the limbic system. The PAG, in turn, indirectly controls pain transmission in the dorsal horn through the RVM. This pathway can exert both inhibitory (blue) and facilitatory (red) control (Adapted from Fields 2004). Abbreviations: ACC, anterior cingulate cortex; H, hypothalamus; PAG, periaqueductal grey ; RVM, rostral ventromedial medulla; T, thalamus.

1.3.2.b Impact of chronic pain on the function of the EOS

Chronic pain is considered a disease state that is mechanistically distinct from acute pain (Tracey and Bushnell 2009). Concretely, chronic pain is defined as pain that persists beyond normal healing time and therefore, has lost its physiological survival function (Treede et al., 2015).

As commented above, the EOS plays an important role in pain modulation and its activation can inhibit pain sensation. However, several studies have shown that **chronic pain correlates with changes in the EOS**. Concretely, in humans, the positron emission tomography (PET) technique has been widely used to measure the availability of ORs in the brain of pain suffering patients. The most used tracer, [^{11}C]diprenorphine, binds to MORs, DORs and KORs*f* with equal affinity, therefore the reported changes cannot be attributed to a concrete receptor type. In these studies, patients under different classes of chronic pain show a **decrease of OR availability** in many **regions involved in pain processing** when compared to healthy subjects. Those regions include the thalamus, PAG, anterior cingulate cortex (ACC), insular cortex and other cortical regions (Jones et al., 2004; Maarrawi et al., 2007; Willoch et al., 2004). However, in human cross-sectional studies, findings cannot be directly attributed to the effect of the pain condition. In turn, the observed reduction in opioid binding could also be caused by the pain opioidergic treatment itself or even represent an intrinsic brain difference in people who might be prone to develop chronic pain.

In addition, studies using animal models have reported a decrease of the antinociceptive effect of MOR agonists in DRG neurons, locus ceruleus, and the spinal cord (Aoki et al., 2014; Hurley and Hammond 2001; Jongeling et al., 2009; Shaqura et al., 2004; Zhang et al., 2004). As animals were not

receiving any analgesic treatment, these **loss of MOR function** is therefore induced **by the persistence of pain**. Unfortunately, it is still not clear which are the neurobiological mechanism of this decrease in OR (or, concretely, MOR) function. One proposed explanation is that the reduced receptor availability is caused by an increase in the release of endogenous opioids that would be occupying the receptors (Porro et al., 1991; Zangen et al., 1998; Zubieta et al., 2001). Another possibility is that pain induces a decrease in the expression of OR. Indeed, several data from animal studies further support this second hypothesis. Concretely, a decrease in both mRNA levels and protein expression of MORs has been found in the DRG and spinal cord of animals under different pain conditions (Obara et al., 2009; Obara et al., 2010; Pol et al., 2006; Zhang et al., 1998).

Besides its role in pain processing, the EOS is also a key component of brain response to addictive drugs and natural reinforcers (see section 1.2). Therefore, it is logical to infer that chronic pain may extent its effects beyond the nociceptive processing system and somehow alter the normal function of the reward system. Unfortunately, until now this hypothesis has only been partially explored. Human PET studies have shown that **chronic pain** correlates with a decrease in OR availability in the striatum and, more precisely, a **decrease in MOR availability in the NAc and amygdala** (Brown et al., 2015; Dossantos et al., 2012; Harris et al., 2007). Also a recent PET study in rats reported less OR binding in similar areas of chronic pain animals (Thomson et al., 2018). In this study, Thomson and collaborators also explored MOR expression in the striatum, that was reduced by the presence of chronic pain. Nevertheless, the concrete mechanism that elicits this pain-induced alterations remains poorly understood.

1.3.2.c Impact of pain on motivational and reward processing

As previously mentioned, the MCLS is responsible for the expression of motivated behavior and reinforcement learning triggered by natural and drug rewards, but also for aversive stimuli encoding (Everitt and Robbins 2005; Koob and Volkow 2016; Salamone 2016). Since chronic pain, as previously described (section 1.3.2.b), is able to change the EOS function and the EOS plays an important regulatory function on MCLS DA neurons activity, it is logical to suppose that chronic pain could have an important impact in MCLS function.

Latest human and animal studies have revealed that **persistent pain** negatively impacts on the motivational and reward processing, via **altering the normal function of the MCLS** (for a recent review see Taylor 2017). Studies in patients undergoing different pain conditions (chronic back pain, neuropathic pain, fibromyalgia, irritable bowel syndrome, headache, complex regional pain syndrome and osteoarthritis) and in rats with experimental pain, have reported functional, anatomical or molecular alterations in this system related to the presence of pain (Seminowicz et al., 2009). These investigations identified extensive changes in gray matter (Geha et al., 2008; May 2008), abnormalities in the white matter connectivity (Geha et al., 2008), and neurochemical modifications in glutamate, opioid and DA neurotransmission (Apkarian et al., 2009; Harris et al., 2007; Wood et al., 2007). Precisely, chronic pain states exhibit a **reduced phasic DA singling** (reviewed in Taylor et al., 2016) as reported by human imagining studies (Loggia et al., 2014) and in *in vivo* recordings (Ozaki et al., 2002; Ren et al., 2015; Taylor et al., 2015). In addition, human fMRI studies have revealed **alterations in cortico-striatal connections** in back pain suffering patients. In this longitudinal study, authors reported that, when pain persisted, brain gray matter density decreased. The

study also showed an increased functional connectivity between the ventral striatum (NAc) and PFC in subjects with persistence of pain (Baliki et al., 2012).

Moreover, pain condition has also been related to **alterations in drug seeking behavior** in preclinical studies (reviewed in Massaly et al., 2016). In fact, opiate-rewarding effects at regular doses were decreased by the presence of pain (Lyness et al., 1989; Narita et al., 2005; Ozaki et al., 2002; Taylor et al., 2015; Wade et al., 2013) This effect appears to be mediated by a decrease in drug-induced DA release. Thus, morphine administration neither increased accumbal DA levels nor induced CPP in pain suffering animals (Narita et al., 2005). Moreover, rats under neuropathic pain showed an altered opioid self-administration profile when compared with the sham group, suggesting that pain was suppressing the positive reinforcing properties of these drugs (Martin et al., 2007), So, initially, the presence of pain could have been considered as a protective factor against the reinforcing effect of opioids. However, another self-administration study reported that, when using higher doses than the ones selected by Martin et al., 2007, rats under inflammatory pain significantly increased their intake only in the case of this higher dose (Hipólito et al., 2015). And the increase was not related to changes in the analgesic effect of the different doses. This observation means that the presence of **pain could promote an escalation** in opioid consumption. In this way, pain-suffering subjects would seek higher doses of opioids to obtain the same DA levels as lower doses of drug provoke in healthy subjects. Unfortunately, the exact mechanism that could be mediating this phenomenon remains unknown. Moreover, it has neither been deeply examined, in a preclinical setting, wether this phenomena also occurs with other drugs of abuse, including alcohol.

Finally, **motivation for natural rewards is also decreased** in animals under pain condition. In this way, reward consumption was not altered when a natural reward was easily available (i.e., sucrose self-administration under a fixed-ratio operant responding task) (Okun et al. 2016; Schwartz et al., 2014). In this type of tasks animals do not need to invest a high amount of energy to perform the task and, therefore, obtain the reward. However, when the required effort increases, pain suffering animals showed less motivation for sucrose pellets (measured as a decrease in the progressive ratio breakpoint, PR) (Massaly et al., 2019; Schwartz et al., 2014). The PR schedule is frequently used in preclinical studies to measure motivation. In this type of studies, animals are required to press the lever a higher number of times (exponentially increasing) to obtain the next sucrose pellet. Concretely, animals under pain conditions showed a lower PR breakpoint, which means that they are less motivated to obtain a natural reward.

The aforementioned data evidence that motivation and reward processes are altered in pain suffering individuals. However, the neurobiological basis of these alterations remain poorly discern. On the one hand, and given the previously described changes induced by pain in the EOS (section 1.3.2.b), it could be possible that pain is directly inducing changes in the local opioidergic control of the DA neurons activity. In this way, the dysregulation of MORs in the MCLS, and, more concretely, in the VTA would induce changes in MORs modulation of VTA DA firing. On the other hand, it is also plausible that alterations in pain-processing pathways could indirectly alter the normal function of DA singling within this reinforcing pathways, without inducing local alterations. Finally, it could also occur that those two mechanisms simultaneously take place, increasing the complexity of pain effect on the MCLS.

1.3.3 EFFECT OF PAIN ON AUD

During the last decades, there has been an increase in data evidencing the interrelation between pain and alcohol abuse (for review see Zale et al., 2015). Concretely, **epidemiological data have reported the co-ocurrence of pain and alcohol use.** Larson and colleagues showed, in a study with persons seeking treatment for AUD, that 75% of patients (from alcohol preferred group) reported moderate-to-severe past-month pain at least once during the 2-year study period (Larson et al., 2007). Moreover, another study performed in older adults showed similar results. In this case, the prevalence of pain among problem drinkers (those that reported one or more drinking problems in the Drinking Problem Index) was higher (43%) that the observed among non-problem drinkers (30%) (Brennan et al., 2005). Furthermore, an interesting correlation can be found in a study from Von Korff and colleagues that investigates comorbidity between chronic back and neck pain and other physical and mental disorders in the USA population. One of their findings was that patients with chronic spinal pain were at significantly increased risk of alcohol abuse and dependence (Von Korff et al., 2005).

It has been reported that the presence of **pain may alter alcohol consumption patterns**. In fact, the presence of persistent pain is directly correlated with an **increment in alcohol consumption and an increased risk of developing an AUD.** Concretely, Witkiewitz and colleagues reported that the percentage of population that developed an AUD increased from 15% in the general population to 40-50% in chronic pain patients (Witkiewitz et al., 2015a). Additionally, greater levels of pain intensity and unpleasantness are associated with increases in alcohol consumption and rates of hazardous drinking (Lawton and Simpson 2009). Evidence also indicates that pain could

act as a stressing factor and, hence, motivate alcohol abuse. Greater levels of alcohol consumption and an increase in the frequency of drinking problems have been detected in patients that report an stress-coping motivation for drinking (Holahan et al., 2001). In this line, patients under both chronic pain and SUDs reported pain as their primary reason for initiating alcohol or drug misuse (Sheu et al., 2008). Despite all this clinical evidence, very few studies have explored pain effect on alcohol consumption in a preclinical setting. The only existing results, though, appear to be in accordance with clinical data, as the presence of pain increased ethanol consumption in non-operant self-administration models in mice (Butler et al. 2017; Yu et al., 2018).

On the opposite, several researches have evidenced that an **excessive alcohol consumption can be related with the appearance of pain problems**. In fact, a long exposure to alcohol undergoes with functional and structural changes of many brain areas, what appears to contribute to the development of chronic pain among persons with AUD (Egli et al., 2012). Another pain problem derived from excessive alcohol consumption is the presence of hyperalgesia, that is an abnormally increased sensitivity to pain. This phenomenon has been reported to appear during abstinence periods in both human and animal studies (Gatch 2009; Jochum et al., 2010). On the opposite, it is important to note that animal and human studies have also showed that alcohol may have a **pain-inhibitory effect** (Ibironke and Oyekunle 2012; Perrino et al., 2008). This analgesic effect of alcohol has been suggested to be mediated by the opioid system (Campbell et al., 2007). However, this would represent a short-term effect, as it tends to diminish following 10-12 days of alcohol administration (Gatch and Lal 1999).

As previously highlighted, relapse constitutes one of the main obstacles that hampers the correct treatment of AUDs (see section 1.3.1). In this line, it is also important to highlight a clinical study showing that **higher levels of pain correlated with a higher risk of relapse** (Jakubczyk et al., 2016). One of the main characteristics of AUD is the abnormal persistence of negative affective states during withdrawal, that can promote drug seeking and relapse (Edwards and Koob 2010). Similar negative affective states are also frequently elicited by pain, driving to alterations in reward evaluation, decision making and motivation (Apkarian et al., 2013) (see section 1.3.2). Therefore, it would be possible that the presence of pain in AUD patients may exacerbate this negative affective state and constitute an important risk factor for relapse. Unfortunately, there is still a lack of studies that further deep in the mechanisms underlying the impact of pain on alcohol relapse.

2 OBJECTIVES

The general aim of the present Thesis is to explore inflammatory pain-induced alterations of the EOS modulation over the dopaminergic MCLS and to investigate wether those alterations could impact on ethanol relapse in animal models. Different experiments have been designed in order to achieve the following concrete objectives:

O1 To investigate the effect of inflammatory pain on MORs function in the MCLS.

O2 To study the neurochemical and behavioral consequences elicited by inflammatory pain on ethanol reinforcing properties.

O3 To explore the effect of inflammatory pain on alcohol relapse, concretely in the ADE in long-term experienced animals.

3 MATERIALS AND METHODS

3.1 ANIMALS

Male albino Wistar rats (Envigo, n=20) were used for long term non-operant ethanol self-administration and Sprague-Dawley (Envigo, n=183) were used for immunohistochemical, neurochemical and behavioral studies, all weighting between 300 and 340 g at the time of surgery. Rats were housed in plastic cages (48 x 38 x 21 cm^3) with controlled humidity and temperature (22°C), a 12/12-h light/dark cycle (on 08:00, off 20:00), and free access to food and water before starting the experiment. After surgery and/or during experiments, rats were housed in individual rectangular plastic cages (48 x 38 x 21 cm^3), located side by side in order to prevent the influence of chronic stress on performance due to isolation, with free access to regular chow and water. All the procedures were carried out in strict accordance with the EEC Council Directive 86/609, Spanish laws (RD 53/2013) and animal protection policies. Experiments were approved by the Animal Care Committee of the University of Valencia and authorized by the Regional Government.

3.2 DRUGS AND CHEMICALS

Artificial Cerebrospinal Fluid (aCSF)

All the experiments with intra-VTA drug administration were performed using aCSF solutions as vehicle. Two kinds of aCSF solution were prepared due to the different needs of the experimental techniques (microdialysis and microinjection procedures). The aCSF solution for microdialysis consisted of 0.1 mM aqueous phosphate buffer containing 147 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$. The pH of the solution was adjusted to 7.4 (Santiago and Westerink 1990). The composition of the aCSF for intracranial microinjections consisted of 120.0 mM NaCl, 4.8 mM KCl, 1.2 mM

KH_2PO_4, 1.2 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 10.0 mM D-glucose and 0.2 mM ascorbate. In this case, the pH of the solution was adjusted to 6.5 (Martí-Prats et al., 2015b).

DAMGO

DAMGO (D-Ala^2, N-MePhe^4, Glyol^5-enkephalin, Sigma Chemical Co) is an OR agonist with specific selectivity for the MOR (Gillan and Kosterlits, 1982). Its chemical structure can be found in Figure 3.1. The DAMGO powder was dissolved in distilled water to obtain a 1 mM solution. This stock solution was kept frozen at -20°C as aliquots until use. Prior to use, aliquots were conveniently diluted with aCSF to the appropriate concentration (see experiment I in section 3.6, and experiment II and experiment III in section 3.7) (Hipolito et al., 2015).

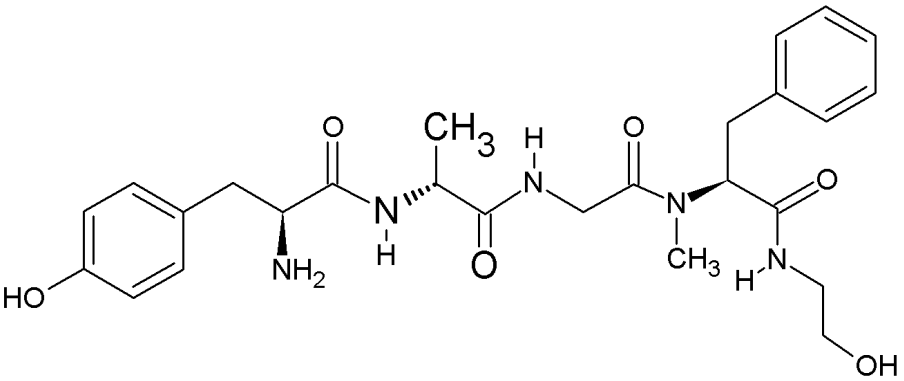

Figure 3.1 DAMGO chemical structure (Molecular Weight: 513.5 g/mol).

β-Funaltrexamine hydrochloride (β-FNA)

Stock solutions of the irreversible MORs antagonist, β-FNA ((E)-4-[[(5α,6β)-17-(Cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-

yl]amino]-4-oxo-2-butenoic acid methyl ester hydrochloride) (Sigma), were prepared by dissolving the compound in the correct volume of distilled water to obtain a 13.6 mM concentration of β-FNA. Aliquots of these solutions were then kept frozen at –20°C until use. Prior to use, aliquots of the stock solutions were conveniently diluted with aCSF solution to the appropriate concentration (8.3 mM) (Sánchez-Catalán et al., 2009). β-FNA hydrochloride chemical structure can be found in Figure 3.2.

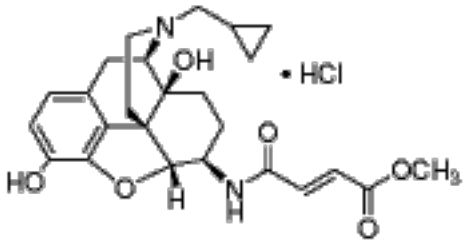

Figure 3.2 β-FNA hydrochloride chemical structure (Molecular Weight: 491 g/mol).

Ethanol

The solution used, 96% v/v ethanol, was purchased from Scharlau. For subcutaneous administration ethanol was conveniently diluted with sterile normal saline to the appropriate concentration (1.5 g/kg in 2 mL). For non-operant self-administration experiment, ethanol was diluted with tap water to three different concentrations (5%, 10% and 20% (v/v)).

Other reagents

All other reagents needed for the different experimental procedures used in the present work were of the highest commercially available grade.

3.3 SURGICAL AND POST-SURGICAL CARE

Rats were anaesthetized with ketamine hydrocloride/xylazine hydrochloride (Esteve veterinary) (80 mg/kg of ketamine and 10 mg/kg of xylazine, i.p.) or isoflurane (Esteve veterinary) (1.5 MAC, inhalatory, VetMet) depending on the experiment. The anesthetic drugs used were changed over the period of time during the course of experiments in order to both reduce animal suffering (e.g., faster recovery with the inhalators anesthesia and more control of effect duration) and to improve the method (e.g., reduce the surgery time). Once deeply anaesthetized, rats were placed in a stereotaxic apparatus (Stoelting, USA). The skin was disinfected with povidone-iodine complex solution (10%) and, following, an incision was made over the skull (8-10 mm), covering the wounds with lidocaine gel (3%) (compounded) (see Figure 3.3). The surgical procedure was necessary for three different experimental protocols (immunohistochemical, microdialysis and CPP studies) that will be described in sections 3.6, 3.7 and 3.8.1.

For **microdialysis experiment II** and **experiment III**, rats were implanted with bilateral vertical concentric-style microdialysis probes into the NAc (anteroposterior: +1.5 mm, mediolateral: ±1.6 mm and dorsoventral: -8.0 mm from bregma, according to Paxinos and Watson 2007). They contained 2 mm of active membrane (Hospal AN69; molecular cutoff 60,000 Da) and were constructed according to Santiago and Westerink 1990 (described in section 3.7.1). Then, probes assemblies were secured in place with dental cement.

For **immunohistochemical** and **CPP** studies, the surgery was performed with the objective of implanting one (for immunohistochemical **experiment I**) or two (for CPP e**xperiment V**, **experiment VI** and **experiment VII**) guide

cannulae for intra-VTA drug administration. In this way, animals were implanted with 28-gauge guide cannulae (Plastics One) aimed at 1.0 mm above the VTA. The coordinates relating to bregma and skull surface (Paxinos and Watson 2007) were as follows: anteroposterior: –6.0 mm; mediolateral: ±1.9 mm; dorsoventral: –7.8 mm. Cannulae were angled toward the midline at 10º from the vertical (all the measurements in the dorsal-ventral plane refer to distances along the track at 10º from the vertical). Cannulae assemblies were secured in place with dental cement. A stainless-steel stylet (33-gauge, Plastics One), extending 1.0 mm beyond the tip of the guide cannula, was put in place at the time of surgery and removed at the time of testing.

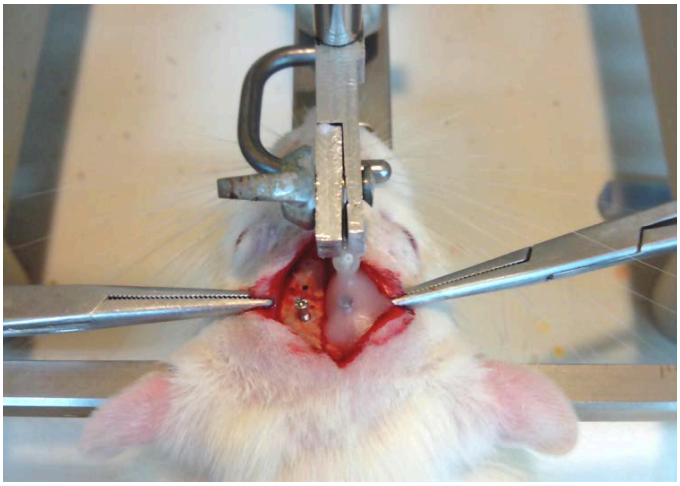

Figure 3.3 Surgical implantation of the microdialysis probe

3.4 INFLAMMATORY PAIN MODEL

The **Complete Freund Adjuvant (CFA)** model of inflammatory pain was selected. The CFA consists of heat-killed Mycobacterium tuberculosis in non-metabolizable oils (paraffin oil and mannide monooleate). CFA (Calbiochem) was diluted in the same volume of sterile saline before its subcutaneous injection (0.1 mL) in the plantar surface of the hindpaw (Hipólito et al. 2015). The CFA injection induces local inflammation, paw swelling, and pain (see Figure 3.4), which persist for at least 2 weeks after injection (Chang et al., 2010).

With the objective to assess the level of inflammation induced by CFA injection, the dorsoventral distance of the rats injected hindpaw was measured and compared to the contralateral hindpaw distance. This measurement was performed right before sacrifice.

For the CPP experiment, we tested the **nociception thresholds** before and after 2 and 9 days of intraplantar injections. Following 20 min of habituation to the apparatus, nociception thresholds were measured by the manual application of five filaments (Aesthesio®) with a simplified up-down method, as described in Bonin 2014 (Bonin et al., 2014). Results were expressed by the mean of nociception threshold (in grams, g).

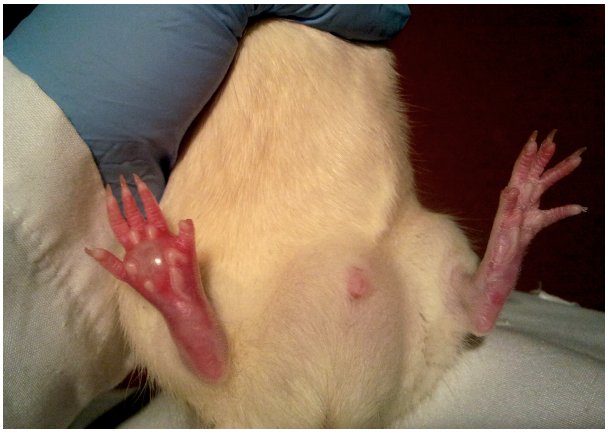

Figure 3.4 CFA inflammatory pain model. CFA treated hindpaw (left) and contralateral (right) 48 hours after the s.c. injection

3.5 MICROINJECTION PROCEDURE

All the intra-VTA drug microinjections were carried out with 33-gauge stainless steel injectors (Plastics One), extending 1.0 mm below the tip of the guide cannulae. Injectors were attached to a 25 mL Hamilton syringe by using PE-10 tubing. Microinjections were performed using a syringe pump (Kd Scientific) which was programmed to deliver a total volume of 200 nL in 20 s (flow rate of 0.6 mL/min) with the exception of the β-FNA injections in which the syringe pump was programmed to deliver a total volume of 300 nL in 2 min (flow rate of 0.15 μL/min). Following the infusion, the injector remained in place for 1.5 min to allow the diffusion of the drugs, and then it was removed. All the injections were carried out in the experimental room.

3.6 IMMUNOHISTOCHEMICAL STUDIES

A immunohistochemical procedure was selected to measure **cFos** expression, an immediate early gene traditionally used as an **indicator of neural activation** (Kovacs 2008). There are several reasons that make cFos the most widely used functional anatomical marker of activated neurons within the central nervous system: (i) at basal condition it is expressed at low levels all over the brain; (ii) several extracellular signals have been shown to induce its expression; (iv) cFos expression (mRNA or protein) detection can be easily carried out; (v) its expression detection can be combined with other markers.

In experiment I, cFos induction was measured in different brain regions to achieve part of the concrete objective O1, investigate the effect of inflammatory pain on MORs function in the MCLS. For that, experiment I was divided in two parts:

i. **Experiment Ia: Study of the effect of the activation of VTA MORs by an agonist (DAMGO) on cFos expression in projection areas.** The different groups planned for this experiment, depending on the VTA treatment, were: saline + aCSF (n=6), saline + DAMGO 7ng (n=6) and saline + DAMGO 14ng (n=6).

ii. **Experiment Ib: Study of the impact of inflammatory pain on the effect of DAMGO intra-VTA injected on cFos expression in projection areas**. The different groups planned for this experiment, depending on the VTA treatment, were: CFA + aCSF (n=6), CFA + DAMGO 7 ng (n= 6) and CFA + DAMGO 14 ng (n= 6).

3.6.1 WORKING SOLUTIONS

The following solutions were used for the perfusion procedure. Phosphate Buffer (PB) and Phosphate Buffer Saline 10x (PBS 10x) were stored at room temperature and Phosphate Buffer Saline 1x (PBS) and Paraformaldehyde (PFA) 4% in PB were freshly prepared before each perfusion.

Phosphate Buffer (PB) 0.4M	
Sodium phosphate dihydrate	13.8 g
Disodium phosphate	42.58 g
Distilled water	q.s. 1 L
pH	7.4

Paraformaldehyde (PFA) 4% in PB 0.1 M	
Paraformaldehyde 37%	100 mL
PB 0.4M	250 mL
Distilled water	q.s. 1 L

Phosphate Buffer Saline 10x (PBS 10x)	
Sodium phosphate dihydrate	3.9 g
Disodium phosphate	10.65 g
Sodium chloride	83 g
Distilled water	q.s. 1 L

Phosphate Buffer Saline 1x (PBS)	
PBS 10x	100 mL
Distilled water	q.s. 1L

Sucrose 30% in PB	
Sucrose	300 g
PB 0.4M	q.s. 1 L

For the immunohistochemical procedure the following solutions were prepared and stored at 4°C until use:

Tris 0.5 M	
Trizma® base	60.55 g
Distilled water	q.s. 1 L

Trizma Buffer (TB) 0.05M	
Tris 0.5M	100 mL
Distilled water	q.s. 1 L
pH	7.6

Trizma Buffer Saline (TBS)	
Sodium chloride	9 g
TB 0.05M	q.s. 1 L

TBS-Tx100 (10%)	
Triton-Tx 100	10 mL
TBS	90 mL

3.6.2 EXPERIMENTAL PROCEDURE

3.6.2.a Perfusion and tissue obtention

48 hours after surgery rats were unilaterally microinjected with 200 nL of aCSF or one of the two DAMGO doses into the VTA. 90 minutes after the drug microinjection, animals were deeply anaesthetized with isoflurane and transcardially perfused through the left ventricle with 200 mL of PBS followed by 300 mL of 4% PFA in PB 0.1M. Following, brain was extracted, maintained in PFA solution for 20 hours at 4°C and then transferred to a 30% sucrose solution in PB for 4 more days at 4°C before freezing them at -80°C until sectioning. Finally, 40 mm sections were obtained with a microtome and collected in 30% sucrose solution in PB in 4 parallel series, as described in Zornoza et al., 2005.

3.6.2.b cFos immunohistochemistry

The immunohistochemistry was the technique selected for the determination and quantification of the cFos protein expression among the brain regions selected for the study. Sections for the different areas were selected according to the rat brain atlas (Paxinos and Watson 2007).

Sections selected were transferred to TBS and sequentially incubated (including 3x5 min TBS rising between incubations) in: **1)** 1% hydrogen peroxide in TBS (30 min), **2)** 5% goat serum (Sigma) in TBS-0.3%TX (90 min), **3)** anti-cFos polyclonal antibody (1:1000, Santa Cruz) overnight at 4ºC, **4)** biotinylated anti-rabbit antibody (1:200; Vector Labs) (120 min), **5)** avidin–biotinylated peroxidase complex (1:200; ABC Elite Kit; Vector Labs) (30 min). Then the reaction was visualized by incubating with diaminobenzidine (SigmaFAST, Sigma) (30 min). Finally, sections were mounted on slides with 0.2% gelatin in TB, dehydrated in alcohols, cleared and coverslipped with Eukitt® for microscopical examination. The schedule of the experiment I is showed in schematic manner in Figure 3.5.

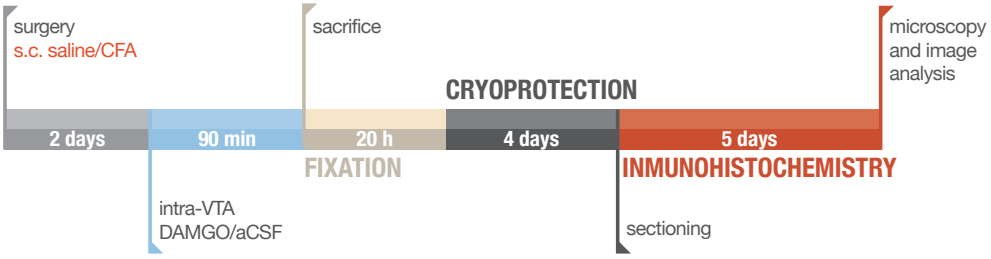

Figure 3.5 Scheme diagram of the experimental procedure experiment I.

3.6.3 IMAGE ANALYSIS

Quantification of the cFos immunoreactive cells (cFos-IR) was performed in the following brain regions: **NAc**, **BLA**, **ACC**, prelimbic cortex (**PL**), infralimbic cortex (**IL**), **BNST** and **VP** (Paxinos and Watson 2007). A schematic diagram of the different brain areas selected for study is shown in Figure 3.6. Two sections per each animal and area were selected and images were digitalized by using a microscope (Leica) equipped with a CCD camera.

The 10x objective was selected to obtain frames of 1026 x 769 mm and the counting of the stained nuclei per frame was carried out using the Multipoint plugin of the software Image J (NIH). The experimenter was blind to experimental grouping throughout images acquisition and processing.

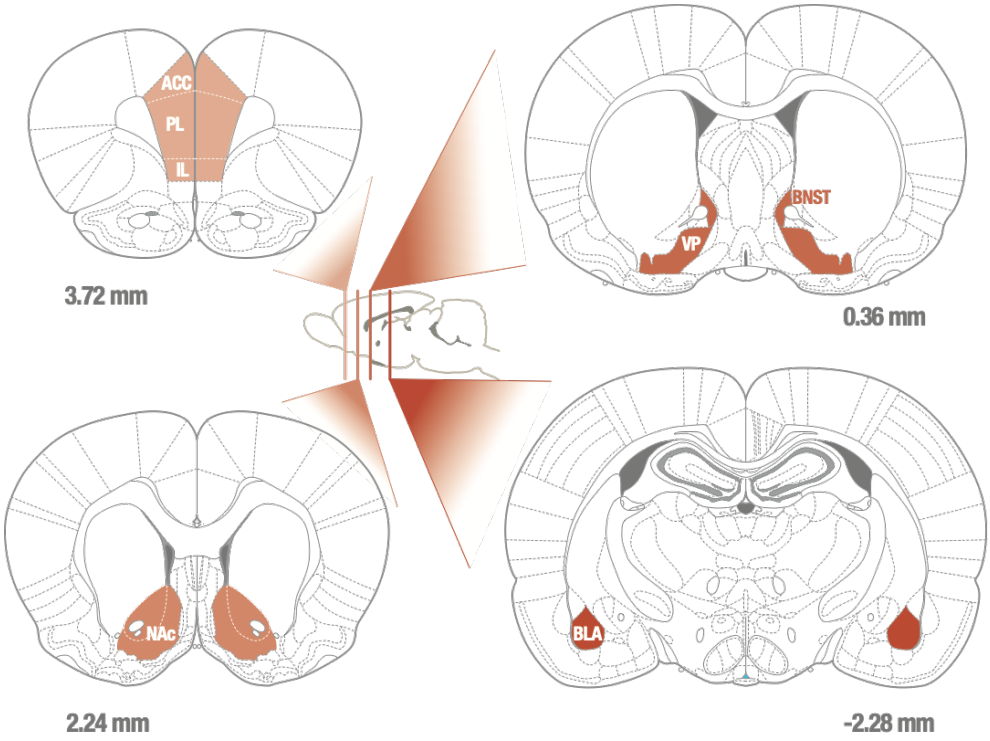

Figure 3.6 Schematic diagram of the brain areas selected for the immunohistochemical study. Numbers indicate distance from anterior to bregma. Abbreviations: ACC: anterior cingulate cortex, PL: prelimbic cortex, IL: infralimbic cortex, NAc: nucleus accumbens, VP: ventral pallidum, BNST: bed nucleus of the stria terminalis, BLA: basolateral amygdala. Adapted from Paxinos and Watson 2007.

3.6.4 DATA ANALYSIS

Data from experiment Ia and experiment Ib were analyzed independently since they were performed at different timing and consequently immunohistochemistry experiment was run separately. After testing for normality with the Shapiro-Wilk test, the average number of cFos-IR under different conditions (experiment, brain area and intra-VTA treatment) was calculated. Data are expressed as mean ± SEM and were analyzed using a one-way ANOVA, followed by Tukey's test. Homogeneity of variance was tested before the ANOVA was performed, and the significance level was always set at p=0.05. When the assumption of the homogeneity of variances was violated, number of cFos-IR were analyzed using Brown-Forsythe test of equality of means, followed by Games-Howell. Differences between dorsoventral measurements of both hindpaw of the CFA-treated animals were analyzed using a t-test for paired samples. Statistical analyses were performed with IBM SPSS statistics 19 software.

3.7 NEUROCHEMICAL STUDIES

The study of the effect of different treatments on the dopamine (DA) levels in NAc of the rats was performed using the *in vivo* microdialysis technique. This method allows us the measurement of the changes in the DA levels in the extracellular space without significant disturbance of the intracellular/extracellular volumes of the brain area studied. Moreover, microdialysis allows the local administration through the dialysis membrane of different pharmacologic agents (retrodialysis application of drug) (Cano-Cebrian et al., 2005). This technique has been set up and validated in our

experimental conditions while it has been continuously used for our group in the last decades.

3.7.1 MICRODIALYSIS PROBES

As it has been explained above, microdialysis allows the continuous measurement and the administration of substances due to the implantation, in a concrete brain area, of a probe provided with a dialysis membrane with permeability for water and some small size molecules depending on the nature of the membrane (Cano-Cebrian et al., 2005). The dialysis membrane is situated between two liquid compartments: the extracellular space and the perfusion liquid (aCSF) that is constantly flowing, in a well-known rate, inside the probe. An interchange of molecules between the extracellular water and the perfusion liquid is taking place through the dialysis membrane as a consequence of the concentrations gradient.

In the present work, DAMGO will reach the brain tissue through the dialysis membrane whereas DA will diffuse inside the probe making possible the sampling of the extracellular levels in real time. These phenomena are schematically represented in the Figure 3.7.

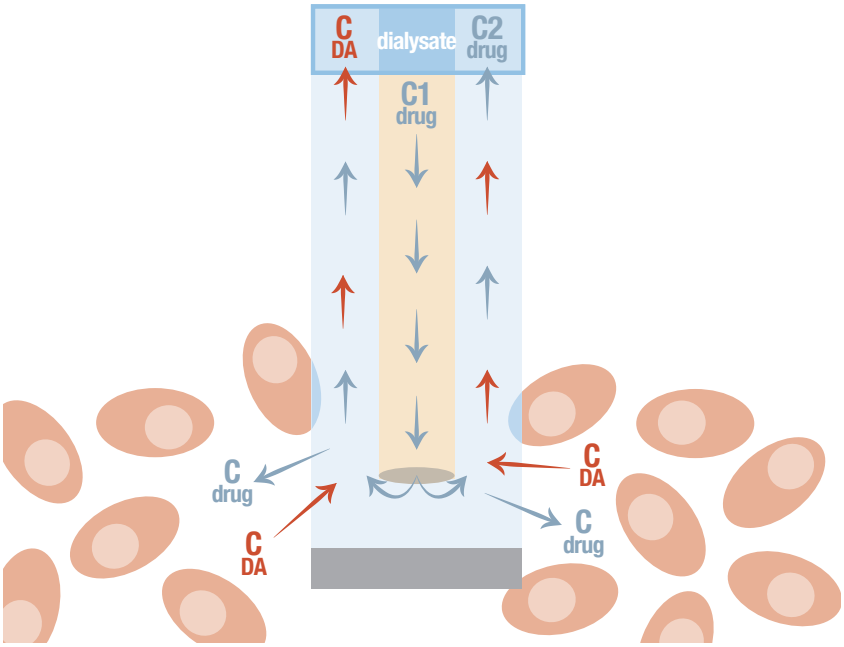

Figure 3.7 Schematic representation of the microdialysis fundament. Abbreviations: C1drug: inflowing drug (for example, DAMGO) concentration, C2drug: outflowing (dialysate) drug concentration (C1>C2), Cdrug: drug concentration that reach the brain tissue, CDA: dialysate DA concentration

The vertical concentric-style microdialysis probes with 2 mm of active membrane were hand-manufactured (Figure 3.8) according to the protocol of Santiago and Westerink 1990. The materials selected for constructing the microdialysis probes were:

- Dialysis membranes: poliacrilonitril membranes Hospal· (AN69), with a 60000 Da molecular cutoff (Bologne, Italy). The inlet diameter size was 220 µm and the outlet 300 µm.
- Silica tube: 75 µm of inlet diameter and 150 µm of outlet diameter.
- Polietilen tube: non-sterile polyethylene tubing (Portex limited, England) with 0.38 mm of inlet diameter and 1.09 mm of outlet diameter.

- Needles: 25 G y 20 mm long needles were used to construct the inlet tube of the probe.
- Cyanoacrylate (Loctite ©) glue and Araldit © and Poxipol © resins were employed.

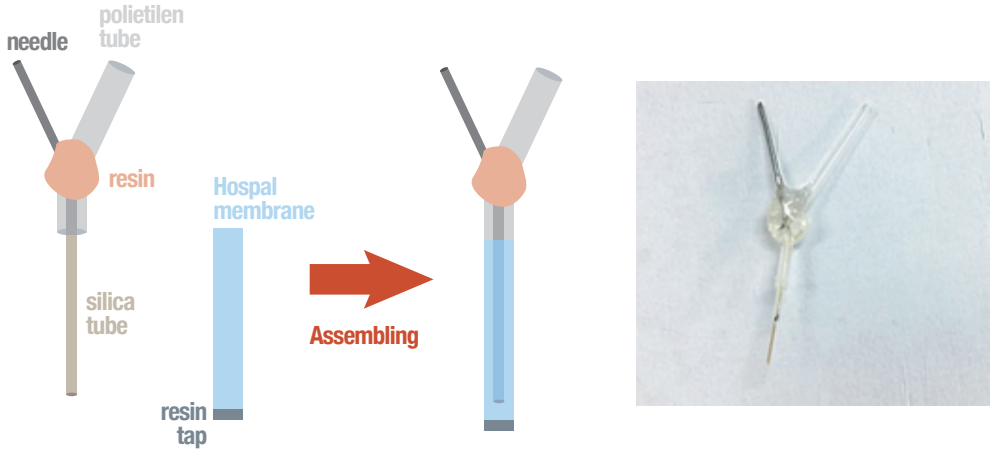

Figure 3.8 Assembling of the microdialysis probe parts (left) and photography of a microdialysis probe (right)

3.7.2 EXPERIMENTAL PROCEDURE

Forty-eight hours after the stereotaxic surgery, animals were placed in Plexiglass bowls. A PE10 inlet tubing was attached to a 2.5 mL syringe (Hamilton) mounted on a syringe pump (Harvard instruments, South Natick, MA, USA) and connected to the probes. Probes were continuously perfused with aCSF at a flow rate of 3.5 µL/min. Following a minimum stabilization period of 1 hour, samples were collected every 20 min and extracellular DA levels were determined by using offline HPLC with electrochemical detection. The HPLC system consisted of a Waters 510 series isocratic pump in conjunction with an electrochemical detector (Mod. Decade, Antec, Leyden,

The Netherlands). The applied potential was = 0.55 V (ISAAC cell, Antec, Leyden, The Netherlands). Dialysates were injected into a 2.1 mm RP-18 column (Phenomenex, Gemini-NX 3 µm, 100 × 2.00 mm) with a 65 µL sample loop. The mobile phase consisted of a sodium acetate/acetic acid buffer (0.05 mol/L, pH = 6) containing 140 mmol/L of sodium chloride, 200 mg/L of 1-octanesulfonic acid, 100 mg/L of EDTA and 150 mL/L of methanol. The mobile phase was pumped through the column at a flow rate of 0.06 mL/min. Chromatograms were analyzed and compared with standards run separately on each experimental day, using the AZUR 4.2 software (Datalys, France). Once the baseline (defined as three consecutive samples with less than 10% of variation) was established, drugs were administered by retrodialysis in NAc, by microinjection into the VTA or by subcutaneous injection depending on the considered experiment. Treatments by retrodialysis were applied for 20 min in order to minimize the concentration gradient around the probe and, therefore, to maximize the anatomical specificity of the pharmacological treatments (i.e., to reduce the spread of the perfused drug to distant sites to the desired brain region). After treatments, DA levels in dialysates were monitored each 20 min for no less than 100 min.

3.7.3 EXPERIMENTAL DESIGN

Microdialysis experiments were conducted in order to achieve part of the two concrete objectives of the present work:

O1, to investigate the effect of inflammatory pain on MORs function in the MCLS. Concretely by testing **pain** modulation of **DAMGO-evoked DA** release over the VTA-NAc pathway.

O2, to study the “neurochemical" and “behavioral” consequences elicited by inflammatory pain on ethanol reinforcing properties. Concretely, by testing **pain** modulation of **ethanol-evoked DA** release in the NAc.

Toward this end three microdialysis studies were planned:

A. **Experiment II: Study of the effect of inflammatory pain on DAMGO-evoked DA release in the NAc.** The DAMGO dose (10 μM), selected from previous work conducted by our group and others (Hipólito et al., 2008; Hirose et al., 2005), was perfused during 20 min through the microdialysis probe into the NAc. A schematic diagram of the procedure can be found in Figure 3.9A. Therefore, two experimental groups (n=5/group) were planned: saline + DAMGO 10 μM and CFA + DAMGO 10 μM

B. **Experiment III: Study of the effect of inflammatory pain on VTA DAMGO-evoked DA release in NAc** Two different DAMGO doses (7ng/200nL or 67ng/200nL) were selected to be administered directly into the VTA with the objective of studying DA release in the NAc. With this purpose 4 experimental groups (n=5/group) were planned: saline + DAMGO 7ng; saline + DAMGO 67ng; CFA + DAMGO 7ng and CFA + DAMGO 67 ng. A schematic diagram of the procedure can be found in Figure 3.9B.

C. **Experiment IV: Study of the effect of inflammatory pain on systemic ethanol-evoked DA release in NAc.** In this experiment ethanol was subcutaneously administered at a dose (1.5 g/kg) that has previously shown to elicit DA release in the NAc (Peters et al., 2017). However, as

acute stressful stimuli can also increase DA levels in the NAc (Abercrombie et al., 1989), in this study we first administered 2 mL of saline (s.c.) and DA levels were analyzed every 20 min for 100 min as a control for the possible effect of the s.c. injection itself. Right after, a single s.c. ethanol dose (1.5 g/kg diluted in 2mL of saline) was administered and DA levels in the dialysates were analyzed for 160 min more. Following this protocol, two experimental groups (n=9/group) were planned: saline + saline + ethanol and CFA + saline + ethanol. Figure 3.9C shows the experimental protocol in a schematic view.

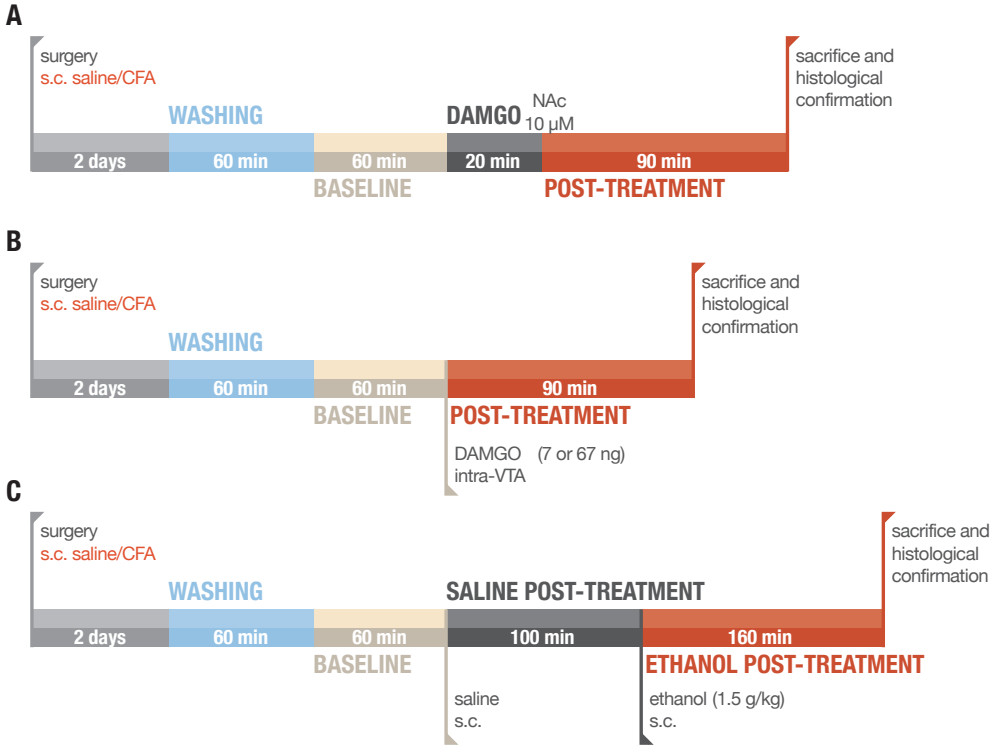

Figure 3.9 Schematic diagram of the experimental procedure in experiment II (A), experiment III (B) and experiment IV (C).

3.7.4 DATA ANALYSIS

In all experiments, basal levels of DA (mean ± SEM) were expressed as fmol in 65 µl. Four basal values were averaged to obtain a basal level for each animal. Differences in basal levels between groups (pain and pain-free animals) were evaluated using the unpaired Student's t-test.

DA levels were also transformed to percentages of baseline (defined as the average value of DA level in three consecutive samples differing in less than 10% in DA content) for each individual rat and were statistically analyzed by a mixed two-way ANOVA for repeated measures, with group (saline or CFA injection) taken as the between factor and time as the within-subjects factor. Significant interaction time x group was further analyzed by means of a Bonferroni correction for multiple comparisons. Significant effects of time in each individual group were analyzed by one-way ANOVA with repeated measures per each group followed by Bonferroni multiple-comparisons test and post-treatment values were compared to the last baseline measure.

In experiment II and experiment III areas under the curve (AUC) were calculated from 0 to 80 min for each rat from percentage data and statistically analyzed by using the unpaired Student's t-test. In experiment IV, AUC values were calculated from 0 to 100 min for the post-saline effect and from 100 to 200 min for the post-ethanol effect for each rat from percentage data. These values were statistically analyzed by mixed two-way ANOVA with repeated measures with group as a between-subjects factor and treatment (saline and ethanol) as a within-subjects factor followed by Bonferroni corrections for multiple comparisons when interactions were found to be significant. In all cases, homogeneity of variance was tested before the ANOVA was performed, and the significance level was always set at $p=0.05$.

3.8 BEHAVIORAL STUDIES

3.8.1 CONDITIONED PLACE PREFERENCE

In the preclinical set up, one of the paradigms widely accepted to approach context-drug associations is the CPP. Rats can develop a preference for an environment due to the association of this context with the reinforcing properties elicited by the drug through its activation of the MCLS. Therefore, it constitutes an appropriate paradigm to study the possible effects of pain on ethanol reinforcing properties and it has also been used to study these pain-induced alterations on opioid mesolimbic activation (Narita et al., 2005; Ozaki et al., 2002). For all that, CPP experiments were planned to achieve the second part of the concrete objective O2, to study the "neurochemical" and "behavioral" consequences elicited by inflammatory pain on ethanol reinforcing properties. And, more precisely, to test the effect of **inflammatory pain** on intra-VTA **ethanol** induced **CPP**. Nonetheless, it is important to highlight that in the previous existing literature there is a lack of CPP studies with focal administration of ethanol into the VTA. Due to this fact, initial experiments were planned in order to characterize the ability of **ethanol** administered directly into the **VTA** to induce **CPP** and to test the role of **MORs** in this behavior.

3.8.1.a Experimental procedure

The CPP test was performed in a home-made two-compartment box connected by a removal barrier with an open door in the middle. The two compartments (30 x 30 x 30 cm) differed by the wall color: black and white vertical stripes (vertical compartment) and black and white horizontal stripes (horizontal compartment) (Figure 3.10). Previous studies performed in our

laboratory with naïve Wistar rats showed no innate preference of rats for any of the two compartments, clearly suggesting the non-biased character of the apparatus. All the experiments were conducted in an isolated room with soft white light illumination.

Figure 3.10 Picture of the CPP apparatus used in the present experiments

Prior and posterior to the surgery, animals were handled every day for 4 days. After recovery of surgery, animals were exposed to the CPP box for 5 min in order to habituate them to the apparatus. The day prior to the conditioning, animal natural preference for the compartments was tested during 15 min (**Pretest**). During the **conditioning** phase, rats received bilateral intra-VTA (aCSF or ethanol) infusions **before** placing them in the compartment associated to the drug or to aCSF administration (time out less than 30 sec). All conditioning phases consisted on 8 sessions (2 sessions/day: morning session and afternoon session) of 30 min distributed in 4 days. Animals were randomly assigned to the experimental or control group and the exposure to conditioning compartments was counterbalanced in both groups. After the last conditioning session, each animal was tested for its place preference (**Test**): the rat was placed in the open door of the barrier, and the time spent in each

compartment was recorded over 15 min. The present protocol has been previously used in our group by Hipólito and collaborators (Hipólito et al., 2011).

As a control for the effect of ethanol administration by itself, an additional group, the so-called ethanol-unpaired control group, was designed. In animals belonging to this group, the association of 70 nmol ethanol administration with the compartment was alternated between days (i.e., horizontal compartment on day 1, vertical compartment on day 2 and so on).

Place preference **scores** were calculated as Test minus Pretest time spent (in seconds) on the ethanol-paired compartment.

3.8.1.b Experimental design

To achieve the previously described objectives the following CPP experiments were conducted:

A. Experiment V: Intra-VTA ethanol dose response for CPP

Following the previously described procedure, 37 rats (n=7-8 per group) were randomly assigned to one of the four experimental groups receiving after a bilateral intra-VTA infusion (20 sec) a total ethanol dose of: 35 nmol, 70 nmol, 150 nmol and 300 nmol. Doses were selected from our previous published experiments covering from low to high doses (Martí-Prats et al., 2013, Sánchez-Catalán et al., 2009). Control group animals (n=7) received 8 administrations (20 sec) of the equivalent volume of aCSF meanwhile the other groups received ethanol or aCSF on alternate sessions (Figure 3.11A). Therefore, all the

planned experimental groups were: aCSF, 35 mol, 70 nmol, 150 nmol, 300 mol and unpaired 70 mol.

B. Experiment VI: MORs role in ethanol CPP acquisition

In this study, 29 animals received two bilateral intra-VTA pre-treatments with 2.5 nmol of the MORs selective antagonist β-FNA or aCSF on the days prior to the first and third conditioning sessions (as previously used in Sánchez-Catalán 2009 (Sánchez-Catalán et al., 2009). Then, during conditioning, rats received either 70 nmol of ethanol and aCSF (experimental group) or only aCSF (control group). (Figure 3.11B). Hence, the different groups planned for this experiment were: aCSF + aCSF, aCSF + ethanol 70 nmol, β-FNA + aCSF and β-FNA + ethanol 70 nmol.

C. Experiment VII: Effect of inflammatory pain on intra-VTA ethanol-induced CPP

In this study, 27 rats (n=5-7 per group) were randomly assigned to one of the two hindpaw treatments (saline or CFA) and one of the two intra-VTA ethanol doses (52 nmol and 70 nmol). Control group animals (n=7) with no hindpaw treatment received 8 administrations of the equivalent volume of aCSF meanwhile the other groups received ethanol or aCSF on alternate sessions (Figure 3.11C). According to that, the different groups planned for this experiment were: control (aCSF), saline + ethanol 52 nmol, CFA + ethanol 52 nmol, saline + ethanol 70 nmol and CFA + ethanol 70 nmol.

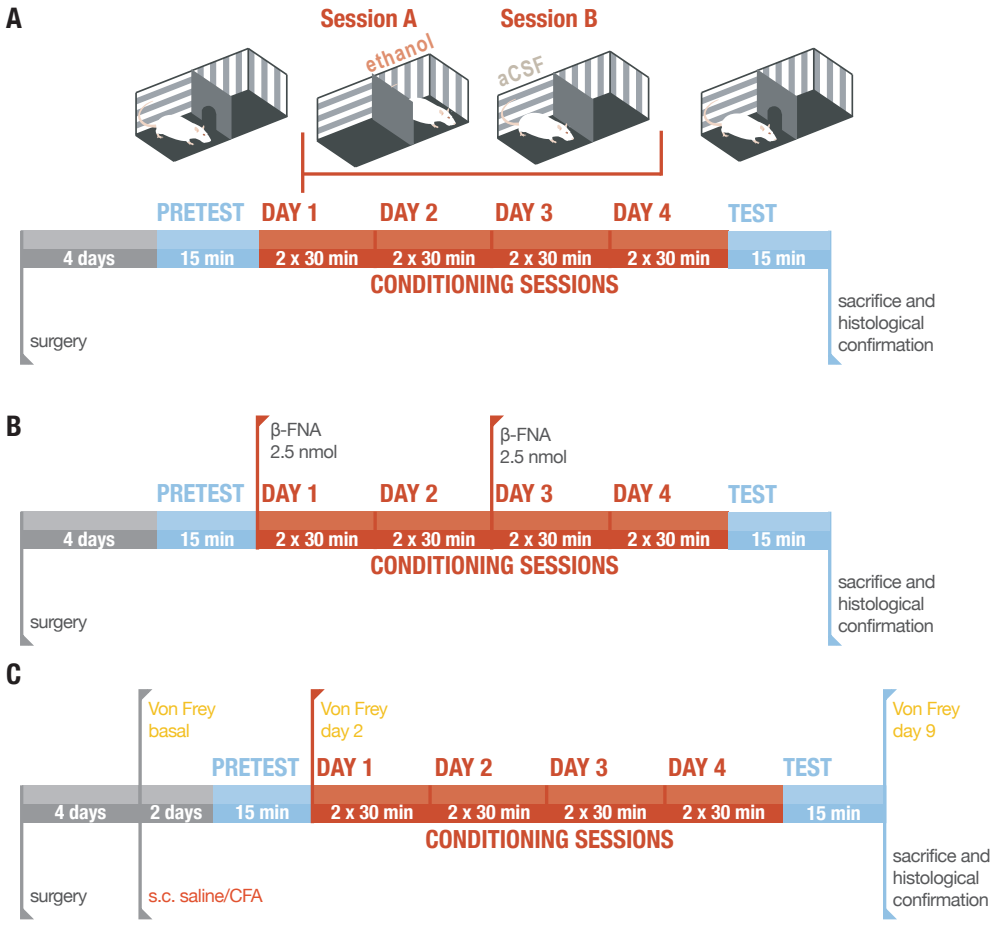

Figure 3.11 Schematic diagram of the experimental procedure for experiment V (A), experiment VI (B) and experiment VII (C).

3.8.1.c Data analysis

Results are expressed in *preference score*, calculated as time spent in ethanol paired compartment during Test minus time spent in the same compartment during Pretest. Preference scores are expressed as mean ± SEM and were analyzed using one-way ANOVA, followed by Tukey's (experiment V

and VI) or LSD (experiment VII) adjustment for multiple comparisons. Homogeneity of variance was tested before the ANOVA, and the significance level was always set at p=0.05. When the assumption of the homogeneity of variances was violated, preference scores were analyzed using Brown-Forsythe test of equality of means, followed by Games-Howell test.

For the Von Frey test results are expressed as mechanical nociceptive threshold in grams. Nociceptive thresholds are expressed as mean ± SEM and were statistically analyzed by mixed two-way ANOVA with repeated measures with group (CFA or saline) as a between-subjects factor and time as a within-subjects factor followed by Bonferroni corrections for multiple comparisons. Homogeneity of variance was tested before the ANOVA, and the significance level was always set at p=0.05.

3.8.2 LONG TERM NON-OPERANT ETHANOL SELF-ADMINISTRATION

With the objective of reproducing the relapse phenomenon we selected the protocol designed by Spanagel and collaborators (Spanagel et al., 1999) and previously set up and validated in our laboratory (Orrico et al., 2013), performing the appropriated modifications to adapt it to our experimental conditions. It constitutes a long-term protocol that includes abstinence periods randomly distributed along the experimental protocol. This alternation allows both to strongly reproduce the relapse phenomenon that occurs after each ethanol reintroduction and to evaluate it through the measurement of the ADE.

3.8.2.a Experimental procedure

In **experiment VIII**, 20 rats were used to examine the effect of inflammatory pain on ethanol relapse. First, rats were habituated to the animal room for two weeks. Next, animals were given continuous access to 4 bottles filled with tap water and 5 %, 10 %, and 20 % (v/v) ethanol solutions in their home cages. These concentrations are selected to mimic the most commonly used alcoholic drinks (beer, wine and spirits). Every week we weighed the animals, renewed all drinking solutions and changed the positions of the four bottles to avoid location preferences. After 8 weeks of continuous ethanol availability, the first 2-week deprivation period was introduced in which rats had only access to the water bottle. Following, rats were given access to alcohol again. Three more deprivation periods were performed in a random manner. The duration of these drinking and deprivation periods was irregular: 6 ± 2 weeks and 2 ± 1 weeks, respectively, in order to prevent behavioral adaptations (Orrico et al., 2013; Vengeliene et al., 2007). The total volume of liquid intake (mL/day) and the total amount of ethanol intake (g/kg/day) were recorded during the whole experiment by weighing the bottles. Animals were randomly assigned to one of the two experimental groups. The baseline drinking for each group was considered as the average of the measurements of the three last days prior to the 4th abstinence period. During this last abstinence period, and 48h before re-introduction of the ethanol solutions, rats received a subcutaneous administration in the hindpaw of 0.1 ml saline or 0.1 mL CFA. After the re-introduction of the ethanol solutions, the daily weighing routine was restored during the three post-abstinence days in order to assess the ADE. A schematic of the procedure can be found in Figure 3.12.

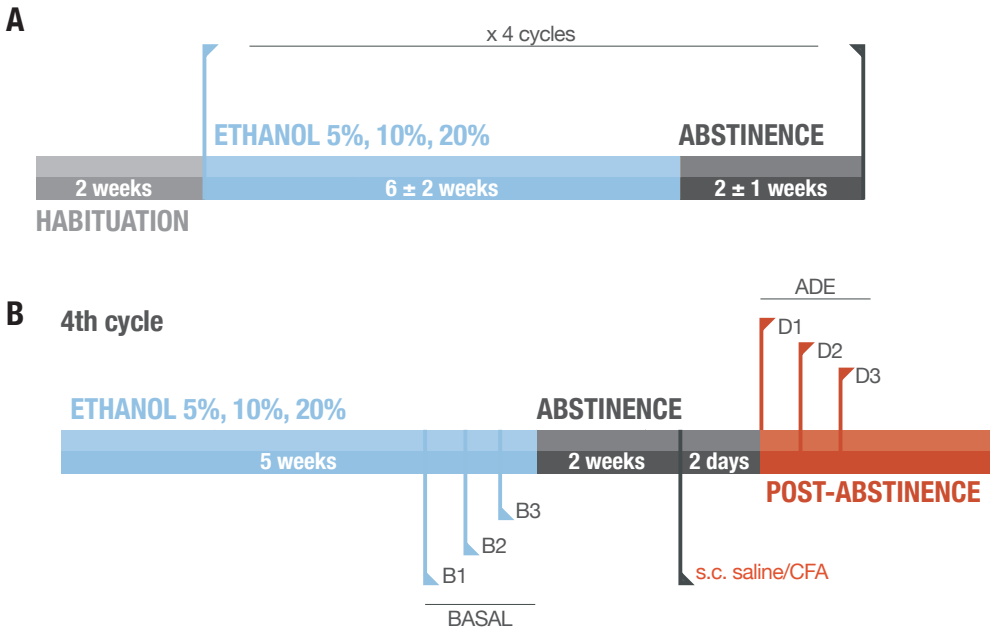

Figure 3.12 Schematic diagram of the experimental procedure

3.8.2.b Data analysis

For the evolution of rats weight and intake of liquids data are expressed as total animal weight (kg) and total volume of liquid (mL/day), respectively. Weekly or daily weight or total volume are expressed as mean ± SEM and were statistically analyzed by mixed two-way ANOVA with repeated measures with group as a between-subjects factor and time as a within-subjects factor followed by Bonferroni corrections for multiple comparisons when interactions were found to be significant.

Experiment data are expressed as total amount of ethanol intake (g/kg/day). Weekly ethanol intake prior to the 4th abstinence period data are

expressed as mean ± SEM and were statistically analyzed by mixed two-way ANOVA with repeated measures with group as a between-subjects factor and period (basal and post-abstinence) as a within-subjects factor followed by Bonferroni corrections for multiple comparisons when interactions were found to be significant. For the analysis of the first three cycles of exposure, basal and post-abstinence ethanol intake are expressed as mean ± SEM and were statistically analyzed by mixed two-way ANOVA with repeated measures with cycle as a between-subjects factor and period (basal and post-abstinence) as a within-subjects. Differences between basal and post-abstinence ethanol intake for each cycle were analyzed by performing a paired t-test. For the analyses of the last cycle, basal and post-abstinence ethanol intake are expressed as mean ± SEM and were statistically analyzed by mixed two-way ANOVA with repeated measures with group as a between-subjects factor and period (basal and post-abstinence) as a within-subjects factor followed by Bonferroni corrections for multiple comparisons when interactions were found to be significant.

In all cases, homogeneity of variance was tested before the ANOVA, and the significance level was always set at $p=0.05$.

3.9 BIOCHEMICAL STUDIES

3.9.1 WESTERN-BLOT

The western blot technique was selected to analyze the expression of the NMDA receptor subunits NR1 and NR2A in the brain tissue obtained from the experiments V and VI.

3.9.1.a Working solutions

The following solutions were used to perform all the western blotting assays:

Homogenizing sucrose buffer

This buffer was used as vehicle for protease inhibitors for the cells lysate and for the protein quantification (see experimental procedure).

Sucrose buffer	
Tris 1M	1 mL
EGTA 0.25M	0.4 mL
$MgCl_2$ 1M	0.05 mL
Sucrose	4.028 g
Distilled water	q.s. 50 mL
pH	7.4

The protease inhibitor mix, purchased from Roche Biochemical, was added to the sucrose buffer at 1% concentration immediately before tissue homogenization.

Loading buffer

This buffer was used as a vehicle to load the samples into the electrophoresis gel. The solution was prepared in a 4x concentration, filtered through 0.2µm pore filter, aliquoted and stored at -20 °C until use.

Loading buffer 4x	
Tris 0.5M	5 mL
Glycerol	4 mL
Sodium Dodecyl Sulphate	0.8 g
β-mercaptoethanol	1 mL
Bromophenol blue	4 mg

Tris/Glycine/SDS (TGS) buffer

This buffer was used to perform the electrophoresis and it was directly purchased from Bio Rad in a 10x concentration and it was diluted in distilled water prior to use. The composition of the TGS 10x buffer was the following:

Tris/Glycine/SDS buffer (TGS) 10x	
Tris	25 nM
Glycine	192 mM
Sodium Dodecyl Sulphate	0.1 %
Distilled water	q.s. 1 L
pH	8.3

Transfer buffer

This buffer was used to carry out the transference from the gels to the nitrocellulose membrane. It was prepared at a 10x concentration and stored at 4°C.

Transfer buffer 10x	
Trizma® base	60.5 g
Glycine	288.4 g
Distilled water	q.s. 2L

Transfer buffer	
Transfer buffer 10x	100 mL
Methanol	200 mL
Distilled water	q.s. 1L

Trizma Buffer Saline Tween 0.1% (TBS-T 0.1%)

This solution was used to wash the membranes between incubations.

Trizma Buffer Saline 10x (TBS 10x)	
Trizma® base	24.4 g
Sodium chloride	80 g
Distilled water	q.s. 1 L
pH	7.6

Trizma Buffer Saline 1x (TBS)	
TBS 10x	100 mL
Distilled water	q.s. 1 L

TBS Tween 0.1% (TBS-T 0.1%)	
Tween 20	1 mL
TBS	q.s. 1 L

5% non-fat dried milk in TBS-T 0.1%

This solution was used to block the nonspecific binding sites in the membrane and to dilute the antibodies for incubation.

5% non-fat dried milk in TBS-T 0.1%	
Non-fat dried milk	2.5 g
TBS-T 0.1%	q.s. 50 mL

Molecular weight marker

The Dual Color Precision Plus Protein Standard (Bio-Rad) was used as a molecular weight marker. This standard provides a migration pattern, with three high-intensity reference bands (25, 50, and 75 kD). 7µL of the standard were loaded into the first well of each gel.

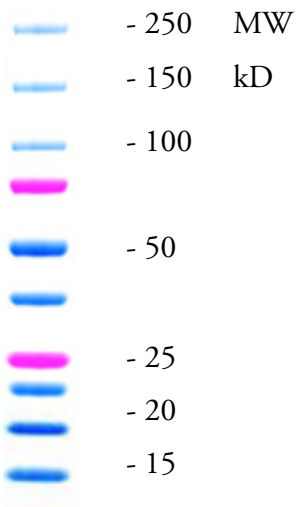

Figure 3.13 Dual Color Standard molecular weight marker code.

Antibodies

All antibodies were diluted to the appropriate concentration in 5% milk TBS-T 0.1%. The primary and secondary antibodies used were

	Antibody	Organism	Dillution	Producer
Primary	NMDA R2A	Rabbit	1:1000	Merck KGaA
	NMDA R1	Rabbit	1:1000	Merck KGaA
	Actine	Mouse	1:1000	Thermofisher
Secondary	HRP-IgG-rabbit	Goat	1:1000	Bio-Rad
	HRP-IgG2b-mouse	Goat	1:1000	Invitrogen

3.9.1.b Experimental procedure

Lysate and protein concentration determination

Serial frontal sections (40 μm) were cut on a cryostat, achieving the brain area to be studied. The anteroposterior stereotaxic coordinates of each area (NAc and hippocampus) were established previously according to the atlas of Paxinos and Watson (Paxinos and Watson 2007). Samples were obtained by punching a portion from an approximately 1-mm-thick coronal slice that included the tested areas. Each of the portions obtained were then homogenized in sucrose buffer containing the protease inhibitor mix by using a dispersing tool (Ultra Turrax T-25 Basic). Thereafter, the homogenates were centrifuged at 13,200× g for 10 min at 4°C to eliminate large cells debris. The supernatant was used as total protein sample.

For protein quantification a standard curve was prepared by diluting a 20g/mL BSA solution (0.5, 0.25, 0.125 and 0.0625 mg/mL). Samples were, then, appropriately diluted in distilled water and both standards and samples were incubated with Bradford Protein Assay reagent (Bio-Rad) for 5 min and the absorbance at 595 nm was measured in a iMark Microplat Reader (Bio-rad). Finally, the protein concentration in each sample was calculated.

Immunoelectrotransference

The appropriate volume of samples was calculated to obtain an equal amount of protein (8 μg for NAc and 15 μg for hippocampus) and together with the loading buffer they were heated for 20 min at 70°C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) gels (4.5% acrylamide stacking gel and 10% acrylamide resolving gel) prepared with the TGX FastCast premixed acrylamide solutions (Bio-rad). Then, proteins were

transferred to 0.45 µm nitrocellulose membranes (Bio-Rad) using a semi-dry transfer system (Trans-Blot Turbo, Bio-Rad) for 30 minutes at 25V. Membranes were then blocked in 5% non-fat dried milk in TBS-T 0.1% and incubated overnight at 4°C with the primary antibody. After three washes in TBS-T 0.1%, the blots were incubated for 1 hour with secondary antibody. Finally, blots were developed using the enhanced chemiluminescence system (Clarity Max ECL, Bio-rad) according to the manufacturer's protocol. Digital images of the immunoblots were obtained in a ChemiDoc Imaging system (Bio-rad) and further analyzed using Image J software (NIH).

3.9.1.c Data analysis

The intensity of the bands was expressed as arbitrary units and normalized by actin expression. The experimental groups were determined by setting the control group (aCSF conditioned animals) to 100% and calculating the respective percentages, expressed as mean ± SEM. Differences in NR1 and NR2A expression levels were analyzed using one-way ANOVA, followed by Tukey's test. Homogeneity of variance was tested before the ANOVA was performed.

3.9.2 RNASCOPE *IN SITU* HYBRIDIZATION

Levels of NR1 and N2A mRNA expression were examined by RNA *in situ* hybridization using the RNAscope® Fluorescent Multiplex Assay (Advanced Cell Diagnostics, ACD). This technique uses a novel and proprietary method of *in situ* hybridization to simultaneously visualize up to three different RNA targets per cell in samples mounted on slides. The assays are based on ACD's patented signal amplification and background suppression technology. Proprietary RNA specific probes are hybridized to target RNA, and are, then, bound to a cascade of signal amplification molecules culminating in signal detection (Figure 3.14).

3.9.2.a Experimental procedure

Tissue obtention

Between 1.5 and 2 hours after the beginning of the test in the CPP experiment, rats were anaesthetized using isoflurane and brains were removed and quickly frozen. Brains were stored at -80°C until sectioning. Following, 15µm sections were obtained by using a cryostat (Leica Biosystems) and they were directly mounted into Superfrost® Plus slides. Slides were then stored for less than 3 months in slide boxes wrapped in air-tight with zip-lock bags at -80°C until use.

RNAscope® Fluorescent Multiplex Assay

First, sections were fixed in 4% PFA in PBS and dehydrated in alcohols. Following and after creating a hydrophobic barrier around each section, a pretreatment with Protease IV (ACD) was applied. Immediately after, the assay was run by performing the following incubations in a regular incubator at 40°C and alternating with two washes (Washing Buffer, ACD): (1) hybridization

with the Rn-Grin-C3 (317021-C3, ACD) (for NR1 mRNA) and Rn-Grin2a (414621, ACD) (for NR2A mRNA) probes, 2 hours; (2) hybridization with Amp 1-FL (ACD), 30 minutes; (3) hybridization with Amp 2-FL (ACD), 15 minutes; (4) hybridization with Amp 3-FL (ACD), 30 minutes; and (4) hybridization with Amp 4-FL-Alt A (ACD), 15 minutes. Finally, slides were counterstained with (4 ',6-diamidino-2-fenilindol) DAPI (ACD) for nuclei staining, coverslipped with Vectashield Mounting Medium for fluorescence (Vector Laboratories) for confocal microscopy examination (Leica Biosystems). A scheme of the assay can be found in Figure 3.14.

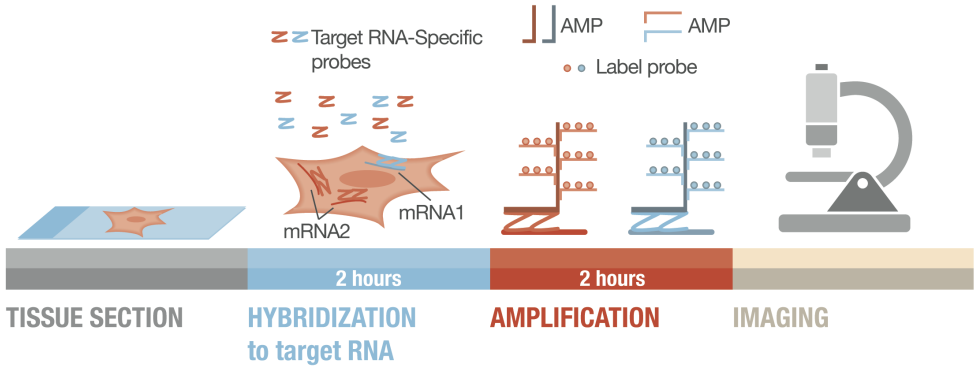

Figure 3.14 Procedure overview. Adapted from the RNAscope® Sample Preparation and Pretreatment Guide for Fresh Frozen Tissue, Part 1 (Document No. 320513-USM).

3.9.2.b Image analysis

The quantification was carried out using the software FIJI (NIH) over merging files composed by 110 or 165 images (NAc and hippocampus, respectively) per hemisphere obtained with a 40x objective. First, for each file, the region of interest was selected (ROI). Next, each fluorophore channel was separately analyzed to obtain the number of signal dots per ROI. For that, the procedure consisted in (1) quantifying the Average Intensity per Single Dot by selecting at least 20 single signal dots and measuring the Area and Integrated Intensity per Single Dot (Total Intensity) (Figure 3.15). The Area of each dot was used to screen whether the dot was a true single dot. Then the Average Intensity per Single Dot was calculated as:

$$Average\ Intensity\ per\ Single\ Dot = \frac{\sum Total\ Intensity\ Single\ Dot}{Number\ Single\ Dots}$$

Following, the Total Dot Number per ROI was calculated by using the Total Intensity per ROI as follow:

$$Total\ Dot\ Number\ per\ ROI = \frac{Total\ Intensity\ per\ ROI}{Average\ Intensity\ per\ Single\ Dot}$$

Finally, the DAPI positive nuclei were counted and used to calculate the Average Dot Number per cell:

$$Average\ Dot\ Number\ per\ cell = \frac{Total\ Dot\ Number\ per\ ROI}{DAPI\ positive\ nuclei\ per\ ROI}$$

As drug administration was bilateral in all the experiments, the measure for each animal was the average resulting from the two-hemisphere counting.

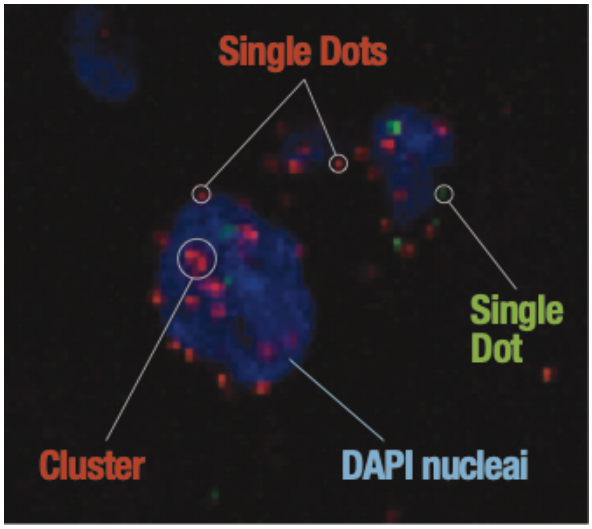

Figure 3.15. Schematic representation of the image quantification. The image was amplified and cropped to facilitate the exemplification of the selection of single dots of Rn-Grin-C3 (NR1 mRNA) in **red** and Rn-Grin2a (NR2A mRNA) in **green** to calculate Average Intensity per Single Dot and the Average Dot Number per cell.

3.9.2.c Data analysis

Experimental data are expressed as mean ± SEM dot number per cell. Differences between groups were analyzed by using an unpaired t-test, and the significance level was always set at p =0.05.

3.10 HISTOLOGY AND PROBE PLACEMENT VALIDATION

A carefully evaluation of the probe and injection cannulae placements was histologically performed at the end of all the experiments. Rats were overdosed with isoflurane and brains were quickly removed and immediately frozen in isopentane. Once the brains were extracted and frozen, they were conserved at -25 °C until histological analysis. To do that, brains were cut in a cryostate (40 μm thick) and slices were collected directly on gelatinized slides. These slides were subjected to a standard Cresyl Violet staining protocol to verify probe and cannulae tip placement under optical microscopy. Paxinos and Watson (Paxinos and Watson 2007) Rat Brain Atlas was used as reference for all probe validations.

4 RESULTS

4.1 EFFECT OF INFLAMMATORY PAIN ON MORs IN THE MCLS

4.1.1 STUDY OF INFLAMMATORY PAIN IMPACT ON cFos EXPRESSION ON VTA-PROJECTING AREAS

4.1.1.a Experiment Ia: Effect of DAMGO injected intra-VTA on cFos expression in projection areas

Cannulae placement validation

At the end of all experiments, a histological evaluation was carefully performed to ensure the placement of the injection cannulae. In experiment Ia, one animal showed cannula placement outside of the VTA. That animal was excluded from the statistical analysis, leaving the DAMGO 7ng group with five animals each, instead of the originally planned six. The tip cannulae placements of the rats included in this experiment are depicted in Figure 4.1.

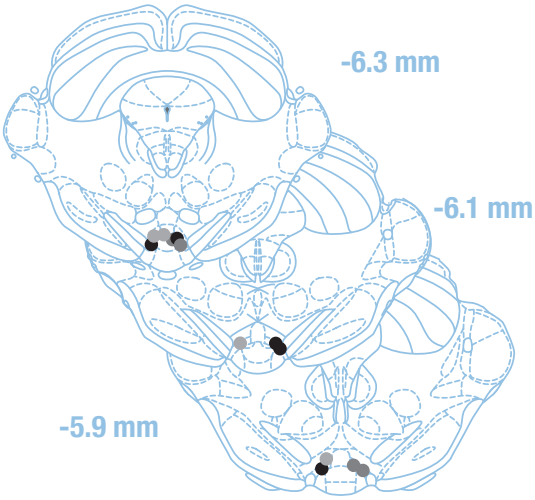

Figure 4.1 Schematic representation of the tip of the cannulae positions in animals belonging to experiment Ia. Experimental groups are: aCSF (**light grey**); DAMGO 7 ng (**dark grey**); and DAMGO 14 ng (**black**). Dots could represent more than one cannula placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

cFos-IR cells counting

In this experiment we evaluated the effect of MORs activation in cFos expression in VTA-recipient areas, by comparing the groups receiving a focal injection of aCSF or DAMGO (7 ng or 14 ng) in the VTA. Data on cFos-IR cells and the corresponding statistical analysis are summarized in Table 4.1. In the **NAc** and **BLA**, the one-way ANOVA detected significant differences between groups ($F_{(2,13)}$=5,180, p=0.022, n= 4-6 and $F_{(2,13)}$=3.894, p=0.047, respectively). The Tukey's test showed that for both areas only DAMGO 14 ng significantly increased cFos expression compared to the aCSF treated group (NAc: 43 ± 10 vs 80 ± 7, p=0.022; BLA: 28 ± 7 vs 47 ± 4, p=0.046), although

a trend to increase the cFos-IR was observed in this two areas when the 7 ng DAMGO was administered (NAc: 43 ± 10 vs 73 ± 10 vs, p=0.108; BLA: 28 ± 7 vs 44 ± 5 IR cells, p=0,140). In **prefrontal regions**, the one-way ANOVA revealed significant differences between groups in the ACC ($F_{(2,14)}$=3.738, p=0.050), but not in PL or IL ($F_{(2,14)}$=0.800, p=0.469 and $F_{(2,14)}$=0.776, p=0.479). Post-hoc analysis showed that cFos-IR was significantly higher in the DAMGO 7 ng group than in aCSF group (141±16 vs 72 ± 20 IR cells, p=0.040). However, no significant differences were found for the 14 ng DAMGO group compared to control (aCSF) or between the two different DAMGO doses (p=0.442 and p=0.305). Similarly, for the **BNST**, the one-way ANOVA showed significant differences between groups ($F_{(2,13)}$=5.036, p=0.024) and the post-hoc analysis revealed that there was a significant difference only between the aCSF and the 7 ng DAMGO groups (p=0.025). Finally, the analysis of the **VP** cFos counting by one-way ANOVA clearly did not show significant differences between groups in cFos-IR ($F_{(2,13)}$=0.021, p=0.979).

All In all, data from experiment Ia showed that the administration of **DAMGO into the VTA significantly increased cFos-IR counting** in the **ACC** and **BNTS** at the dose of **7 ng** and in the **BLA** and **NAc** at the dose of **14 ng**.

Area	VTA treatment		
	aCSF	DAMGO 7ng	DAMGO 14ng
BLA	28 ± 7	44 ± 5	**47 ± 4** *
NAc	43 ± 10	73 ± 10	**80 ± 7** *
ACC	72 ± 20	**141 ± 16** *	103 ± 16
PL	162 ± 32	213 ± 32	179 ± 20
IL	157 ± 20	189 ± 23	165 ± 11
BNST	24 ± 4	**48 ± 8** *	30 ± 3
VP	19 ± 5	20 ± 3	19 ± 2

Table 4.1 Effect of DAMGO injected intra-VTA on cFos expression in projection areas in experiment Ia. cFos-IR cells counting per frame after the administration of aCSF (n= 5-6), DAMGO 7 ng (n= 4-5) or DAMGO 14 ng (n= 5-6) intra-VTA in experiment Ia. Data are expressed as mean ± SEM. Bold font and *: $p \leq 0.05$ compared with aCSF treated group by Tukey's test.

4.1.1.b Experiment Ib: Impact of inflammatory pain on the effect of DAMGO injected intra-VTA on cFos expression in projection areas

CFA-induced inflammation validation

The dorsoventral measurements of the rats hindpaw injected with CFA were significantly different from the non-treated hindpaw confirming the presence of inflammation (CFA treated paw 7.5 ± 0.2 mm vs. non-treated paw 4.1 ± 0.1 mm, t-test for paired samples p< 0.001, Figure 4.2)

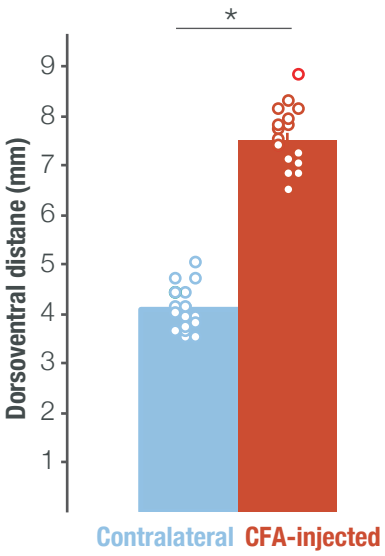

Figure 4.2 Validation of CFA-induced inflammation in the hind paw Dorsoventral distance (mm) of both contralateral (blue) and CFA-injected (red) hind paws of inflammatory pain animals (n=17, *: p<0.001, t-test for paired samples).

Cannulae placement validation

One animal in this experiment showed cannula placement outside of the VTA. That animal was excluded from the statistical analysis, leaving the 7 ng of DAMGO receiving group with five animals each, instead of the originally planned six. The tip cannulae placements of the rats included in this experiment are depicted in Figure 4.3.

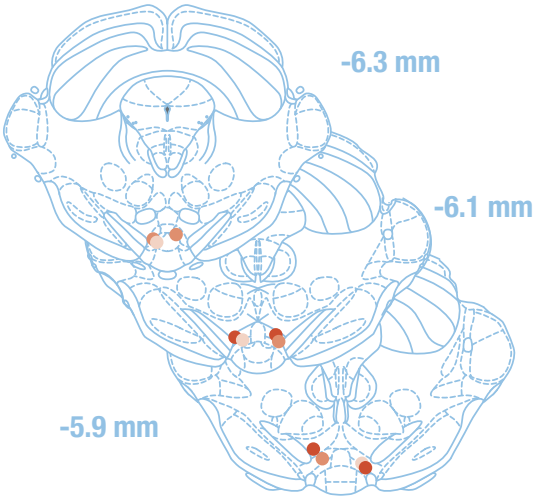

Figure 4.3 Schematic representation of the tip of the cannulae positions in animals belonging to experiment Ib (CFA-treated). Experimental groups are: aCSF (**light orange**); DAMGO 7 ng (**dark orange**); and DAMGO 14 ng (**red**). Dots could represent more than one cannula placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

cFos-IR cells counting

In this second experiment, we evaluated how inflammatory pain affected the previously described MORs activation pattern by comparing the expression of cFos following the administration of DAMGO intra-VTA in rats administered with CFA in the hind paw (Table 4.2).

Regarding the **NAc**, the Brown-Forsythe test showed significant differences for the cFos-IR cells counting (p=0.023) concretely between the highest dose treated group, 14 ng of DAMGO, and aCSF treated group (46 ± 13 vs 93 ± 3; p=0.011). By contrast, after the administration of 7 or 14 ng of DAMGO none of the other areas of the study showed a significant increase of the cFos-IR cell counting. Indeed, the statistical analysis performed for each area failed to find significant differences between groups: **BLA** (one-way ANOVA $F_{(2,13)}$=0.904, p=0.429); **ACC** (Brown-Forsythe test p=0.508); **PL** (one-way ANOVA $F_{(2,14)}$=0.995, p=0.394); **IL** (one-way ANOVA $F_{(2,14)}$=0.075, p=0.929); **BNST** (one-way ANOVA $F_{(2,13)}$=0.458, p=0.642); **VP** (one-way ANOVA $F_{(2,13)}$=0.365, p=0.701). All together, these data showed that, in **CFA rats**, **DAMGO administered into the VTA does not increase cFos-IR counting in the areas that were previously activated in experiment Ia, except from the NAc.**

Area	VTA treatment		
	aCSF	DAMGO 7ng	DAMGO 14ng
BLA	25 ± 6	38 ± 9	39 ±5
NAc	46 ± 13	67 ± 11	**93 ± 3** *
ACC	99 ± 38	120 ± 19	74 ± 15
PL	148 ± 33	209 ± 18	175 ± 33
IL	148 ± 23	161 ± 31	150 ± 22
BNST	29 ± 4	36 ± 7	32 ± 2
VP	19 ± 4	21 ± 7	31 ± 4

Table 4.2 Effect of DAMGO injected intra-VTA on cFos expression in projection areas in CFA rats from experiment Ib. cFos-IR cells counting per frame after the administration of aCSF (n=5-6), DAMGO 7 ng (n=4-5) or DAMGO 14 ng (n=5-6) intra-VTA in experiment Ib. Data are expressed as mean ± SEM. Bold font and *: p≤0.05 compared with aCSF treated group by Tukey's test.

4.1.2 STUDY OF INFLAMMATORY PAIN IMPACT ON DAMGO-EVOKED DOPAMINE RELEASE OVER THE VTA-NAc PATHWAY

4.1.2.a Experiment II: Effect of inflammatory pain on local DAMGO-evoked DA release in the NAc

Probe placement validation

The probe placements within the NAc were carefully confirmed by histological analysis. Figure 4.4 schematically depicts the location of the active portion of the microdialysis probes in the NAc for all the rats included in the present study. Only the data of the experiments in which probes were correctly placed were included in the statistical comparisons.

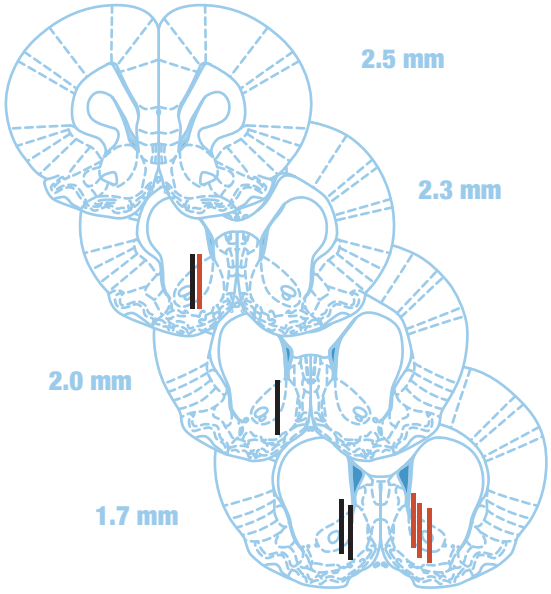

Figure 4.4 Schematic representation of the active membrane of the microdialysis probes into the NAc in animals belonging to experiment II. Experimental groups are: saline rats (**black**) and CFA rats (**red**). Plotted lines could represent more than one probe placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

DA baseline levels

Baseline DA levels (mean ± SEM), calculated from a standard curve, were 71.8 ± 18.9 fmol/65µl and 73.1 ± 13.1 fmol/65µl for the pain-free and pain groups, respectively. These results indicate that the presence of inflammatory pain does not have a significant effect on basal extracellular DA levels (n=10, t-test, p=0.957).

Effect of DAMGO application in the NAc on DA levels

As expected (Hipólito et al., 2008), in **saline rats**, the retrodialysis administration of **DAMGO (10 µM) in the NAc triggered a significant increase in DA release up to 140% form 60 min to 80 min** (one-way ANOVA, $F_{(10,50)}$=5.044; p<0.001, Bonferroni multiple comparisons versus the last basal time point, t=0 min: p_{60}=0.046 and p_{80}=0.043; Figure 4.5, left). However, the **effect of DAMGO was reduced in CFA rats**. Although intra-NAc DAMGO triggered an increase up to 118% from baseline, **no significant differences were found with respect to baseline** (one-way ANOVA, $F_{(10,50)}$=1.937; p=0.062) **or compared to saline rats** (mixed two-way ANOVA, between-subjects effect of group: $F_{(1,10)}$ =3.542, p=0.089, within-subjects effect of time: $F_{(10,100)}$=6.288; p<0.001, interaction time x group: $F_{(10,100)}$=1.064; p=0.397; Figure 4.5, left).

To further study the effect of DAMGO on DA release in both pain and saline rats, we calculated the **AUC between 0 and 80** min for each group (Figure 4.5, right). This measure allowed us to compare the total effect on extracellular DA levels in the NAc, that was **not significantly different** between pain-free group and pain group (n=10, t-test p=0.148).

Overall, results from experiment II showed that the **effect of local DAMGO 10 µM administration into the NAc on DA extracellular levels is not statistically different between the CFA and the saline groups**.

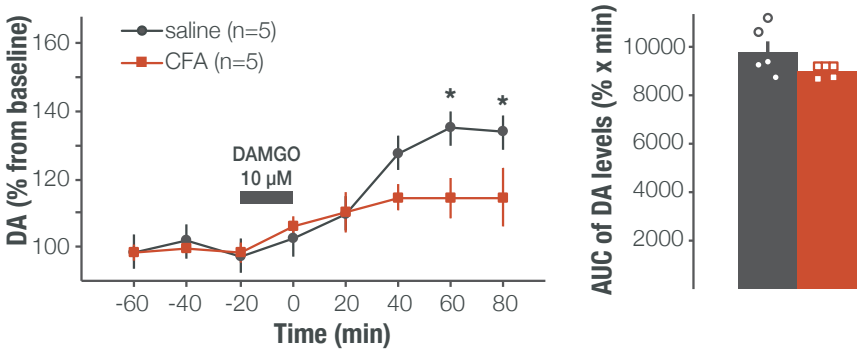

Figure 4.5. Effect of DAMGO 10µM infused in the NAc on DA extracellular levels in the NAc. Left panel: Data are mean ± SEM represented as percentage from baseline DA levels in **black dots** for saline rats and **red squares** for CFA rats (n=5 per group). *=p<0.05, significant differences in the within-subjects effect of time for animals treated with saline (Bonferroni multiple comparisons). Right panel: Data are mean ± SEM of AUC from 0 to 80 min, in **black** for saline rats and **red** for CFA rats (n=5 per group).

4.1.2.b Experiment III: Effect of inflammatory pain on intra-VTA DAMGO-evoked DA release in the NAc

Probe and cannulae placement validation

The probe and cannulae placements within the NAc and VTA were carefully confirmed by histological analysis. Figure 4.6 schematically depicts the location of the active portion of the microdialysis probes in the NAc (left) and the tip of the injection cannulae in the VTA (right) for all the rats included in the present study. Only the data of the experiments in which both probes and cannulae were correctly placed were included in the statistical comparisons.

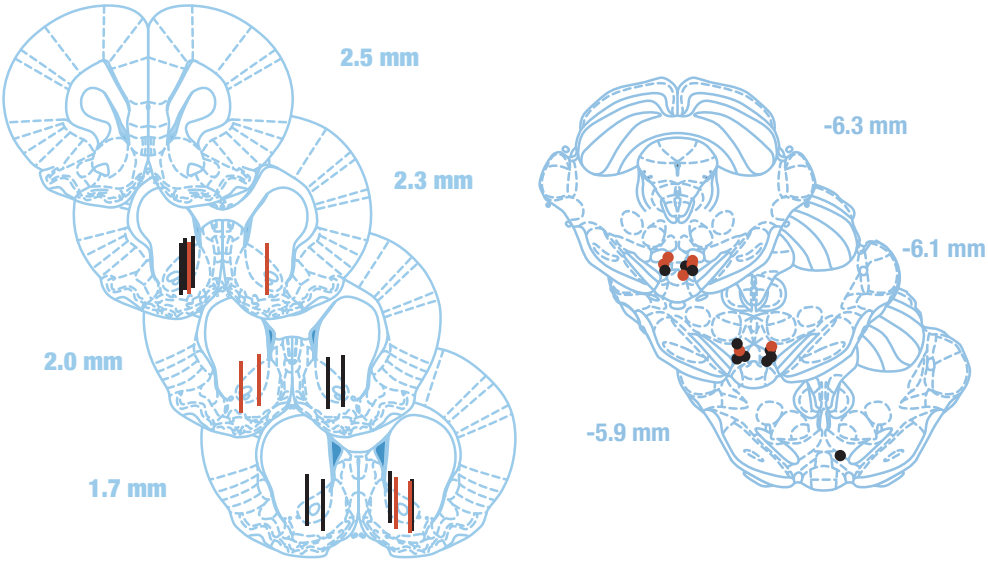

Figure 4.6 Schematic representation of the active membrane of the microdialysis probes into the NAc (left) and cannula tips into the VTA (right) in animals belonging to experiment III. Experimental groups are: saline rats (**black**) and CFA rats (**red**). Lines and dots could represent more than one probe or cannula placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

DA baseline levels

Baseline DA levels (mean ± SEM), calculated from a standard curve, were 61.1 ± 15.4 fmol/65µl and 66.3 ± 11.4 fmol/65µl for the saline and CFA rats, respectively. These results indicate that the presence of inflammatory pain does not have a significant effect on basal extracellular DA levels (n=10, t-test, p=0.793).

Effect of DAMGO application in the VTA on DA levels in the NAc

As shown in Figure 4.7 (upper panel), intra-VTA administration of **7 ng DAMGO elicited a significant release of DA in the NAc in saline rats**, and this effect was **blunted in CFA rats**. Indeed, mixed two-way ANOVA analysis detected differences between the two groups (between-subjects group effect: $F_{(1,8)}$=12.133, p=0.008 and interaction time x group: $F_{(7,56)}$=3.241, p=0.006), and also a significant effect of time (within-subjects effect of time: $F_{(7,56)}$= 16.855, p<0.001). **Saline rats showed a significant increase in DA release in the NAc up to 125%–140% from baseline**, which was initiated 20 min following DAMGO administration and lasted until the end of the experiment (Bonferroni multiple comparisons versus baseline, p_{20}=0.044, p_{40}=0.018, p_{60}=0.018, p_{80}=0.004). In **CFA rats**, the administration of DAMGO intra-VTA induced a much **smaller increase in DA release that was not significantly different** from the respective baseline (Bonferroni multiple comparisons versus baseline from 20 to 80 min p>0.05). This loss of DAMGO effect was especially relevant 20 and 40 min following the administration of DAMGO locally in the VTA (mixed two-way ANOVA, Bonferroni multiple comparisons between groups, p_{20}=0.044 and p_{40}=0.004 vs saline rats).

In order to deeply investigate the plausible desensitization of the MOR in the VTA in CFA rats, we administered a close to **10x higher dose of DAMGO (67 ng)** in the VTA and monitored the DA release in the NAc. Results are shown in Figure 4.7 (lower panel). As can be seen, **both saline and CFA rats experienced a similar and significant increase of DA extracellular levels from baseline after DAMGO administration** (mixed two-way ANOVA within-subjects effect of time: $F_{(7,56)}$=18.392, p<0.001). When analyzing individually, DAMGO 67 ng increased DA levels compared to baseline at time 40 min and 60 min in saline rats (Bonferroni multiple comparisons from

baseline: p_{40}=0.013 and p_{60}=0.032) and at time 40 min in CFA rats (Bonferroni multiple comparisons from baseline: p_{40}=0.018). More interestingly, the mixed two-way ANOVA analysis did not detect differences between the two groups (between-subject effect of group: $F_{(1,8)}$=0.241, p=0.636, and interaction time x group: $F_{(7,56)}$=0.488, p=0.839), supporting the notion that the increase in the DAMGO dose reverses the pain-induced effects.

To further study the effect of the two doses of DAMGO on DA release in both CFA and saline rats, we calculated the **AUC between 0 and 80 min for each group** (Figure 4.7, upper right panel for 7 ng dose and lower right panel for 67 ng dose). This measure allowed us to compare the total effect on extracellular DA levels in the NAc. In accordance with the individual analysis, the **total increase induced by DAMGO 7 ng was significantly higher in saline rats compared to CFA rats** (n=10, t-test p=0.007), whereas **no differences were found** when the **67 ng DAMGO** dose was administered (n=10, t-test p=0.700).

As a whole, experiment III showed that the **effect of intra-VTA DAMGO 7 ng on accumbal DA levels is blunted in CFA rats**. Interestingly, when using a higher dose of **DAMGO (67 ng) there are no statistical differences in DA levels the NAc between saline and CFA rats**.

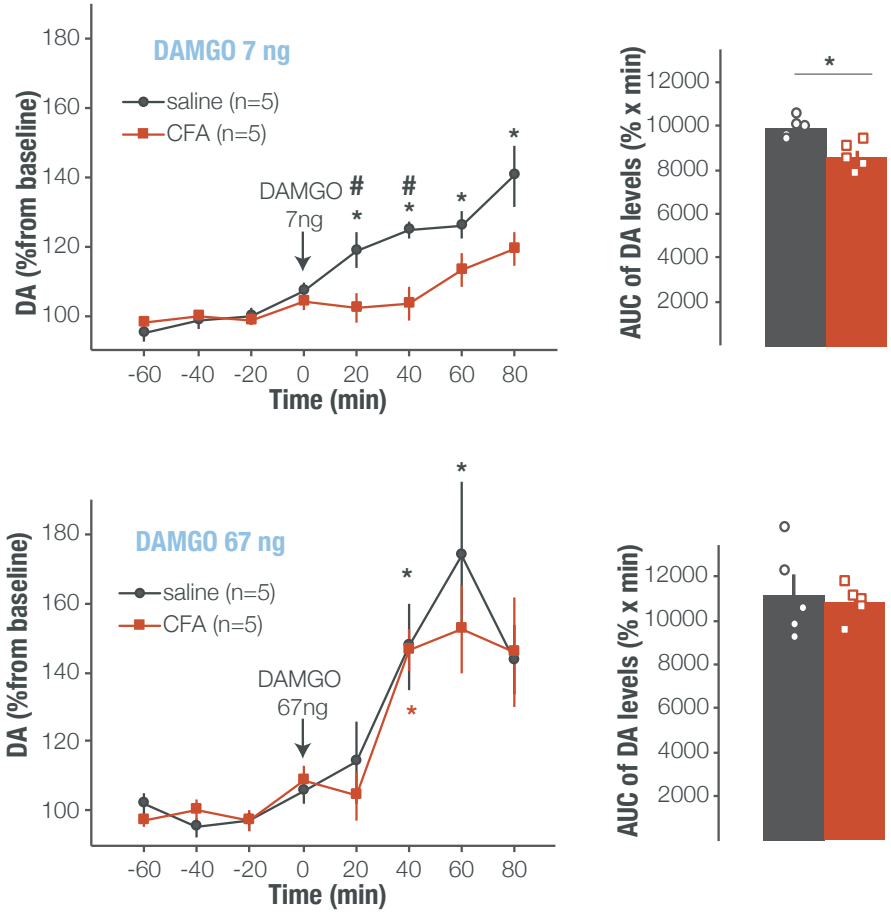

Figure 4.7. Effect of DAMGO administered into the VTA on DA extracellular levels in the NAc. Left panels: Data are mean ± SEM represented as percentage from baseline in **black dots** for saline rats group and **red squares** for CFA rats; n=5 per group. #=p<0.05, significant differences between groups at the time points indicated (Bonferroni multiple comparisons); * and *=p<0.05, significant differences at the indicated points relative to the respective baseline (Bonferroni multiple comparisons). Right panels: Data are mean ± SEM of AUC from 0 to 80 min; in **black** for saline rats and **red** for CFA rats; n=5 per group; *=p<0.05, significant differences (t-test).

4.2 EFFECT OF INFLAMMATORY PAIN ON ETHANOL REINFORCING PROPERTIES

4.2.1 STUDY OF INFLAMMATORY PAIN IMPACT ON SYSTEMIC ETHANOL-EVOKED DA RELEASE IN NAc

Experiment IV

Probe placement validation

The probe placements within the NAc were carefully confirmed by histological analysis. Figure 4.8 schematically depicts the location of the active portion of the microdialysis probes in the NAc for all the rats included in the present study. Only the data from animals in which probes were correctly placed were included in the statistical comparisons.

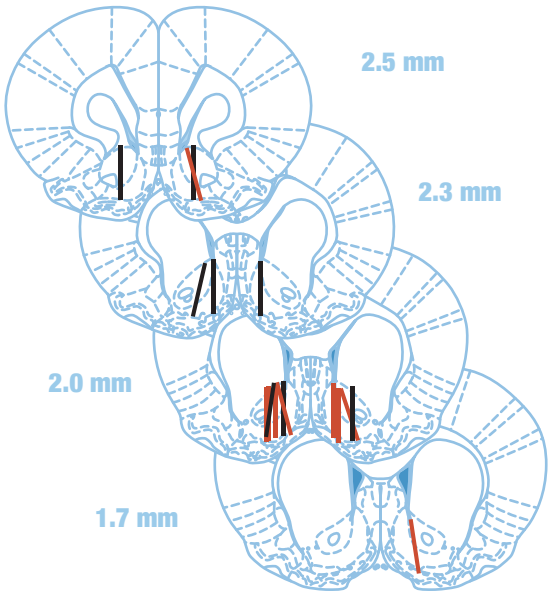

Figure 4.8 Schematic representation of the active membrane of the microdialysis probes into the NAc in animals belonging to experiment IV. Experimental groups are: saline rats (**black**) and CFA rats (**red**). Lines could represent more than one probe placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

DA baseline levels

Baseline DA levels (mean ± SEM), calculated from a standard curve, were 51.8 ± 9.8 fmol/65 µl and 62.8 ± 8.4 fmol/65 µl for the saline and CFA rats, respectively. Our results indicate that the presence of inflammatory pain does not have a significant effect on basal extracellular DA levels (n=17, t-test, p=0.517).

Effect of ethanol subcutaneous administration on DA levels in the NAc

The objective of this experiment was to analyze whether inflammatory pain affects ethanol-evoked DA release within the NAc. Once a stable baseline for DA was achieved (<10% variation in three consecutive samples), rats received a first saline s.c. injection to discriminate the possible effect of the manipulation/injection itself on the NAc DA levels. Next, a single ethanol dose was administered (1.5 g/kg, s.c.) and DA levels were monitored until the end of the experiment for a total of 260 min.

The mixed ANOVA for repeated measures detected a **significant effect in the within-subject variable**. Indeed, both the time ($F_{(16,240)}$= 4.195, $p<0.001$), in the interaction time x group ($F_{(16,240)}=3.506$, $p<0.001$), but not in the effect of group ($F_{(1,15)}=1.140$, $p=0.302$). The analysis of the effect in the within-subject variable time was further analyzed by means of a one-way ANOVA for repeated measures. As can be seen in Figure 4.9, **in saline rats 1.5 g/kg ethanol dose induced a significant increase in DA release up to 135% from 80 min to 120 min** (180 to 220 min time points in Figure 4.9) after its administration (one-way ANOVA for repeated measures, within-subjects effect of time $F_{(16,112)}=7.631$, $p<0.001$; Bonferroni multiple comparisons versus the last basal time point, t=0 min: $p_{180}=0.023$, $p_{200}=0.017$, $p_{220}=0.015$). Interestingly, although **CFA rats** experienced an ethanol-induced DA release (136% from baseline), this **increase was not significantly higher compared to last value of baseline** (one-way ANOVA for repeated measures, within-subjects effect of time $F_{(16,128)}=3.081$ $p=0.0001$, Bonferroni multiple comparisons versus t=0 min: $p<0.001$, from 20 to 260 min $p>0.05$). Indeed, ethanol-evoked DA levels in CFA rats were significantly lower than in saline rats at 60, 80 and 100 min after 1.5 g/kg ethanol administration (Figure 1B;

two-way ANOVA for repeated measures, interaction time x group $F_{(16,240)}=3.506$, $p<0.001$; Bonferroni correction for multiple comparisons, $p_{60}=0.048$, $p_{80}=0.001$, $p_{100}=0.002$). Finally, it is important to mention that saline s.c. injection induced a slight increase up to 127% and 119% in DA release (at 20 min time point) both in CFA and saline rats, respectively, although this increase in DA levels was no statistically significant (CFA rats: $p_{20}=0.688$, saline rats: $p_{20}=0.896$; compared to last baseline point t=0).

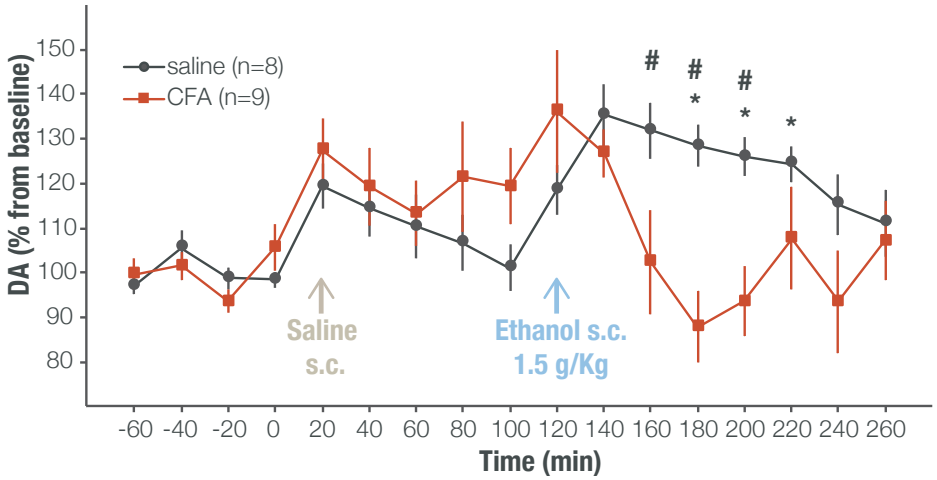

Figure 4.9 Effect of subcutaneous administration of saline and 1.5 g/kg of ethanol on DA extracellular levels in the NAc. Data are mean ± SEM represented as percentage from baseline in **black dots** for saline rats and **red squares** for CFA rats; n=8/9 per group. #=p<0.05, significant differences between groups at the time points indicated (Bonferroni multiple comparison). *=p<0.05, significant differences relative to the respective baseline (t=0) (Bonferroni multiple comparisons).

To further quantify this inflammatory pain-induced effect on the ethanol-evoked DA release, we calculated the AUC from the above curves of DA level vs time for the following 100 min after each administration (saline or ethanol) in both CFA and saline rats (Figure 4.10). **Mean AUC values were significantly different between saline and CFA rats** (two-way ANOVA for repeated measures, within-subjects interaction treatment x group $F_{(1,15)}=6.384$, $p=0.023$). Moreover, the **ethanol-induced total effect in the saline rats was significantly higher than the total effect induced by the saline injection** (Bonferroni correction for multiple comparisons, saline (0-100 min) versus saline (100-200 min): $p=0.013$), whereas **no significant differences were found between saline and ethanol treatments in CFA rats** (Bonferroni correction for multiple comparisons, CFA (0-100 min) versus CFA (100-200 min): $p=0.502$). Finally, when comparing between groups, the ethanol-induced total effect showed a tendency to be higher in saline rats compared to CFA rats, whereas no significant differences were found between saline-induced total effect (Bonferroni correction for multiple comparisons, saline (100-200 min) versus CFA (100-200 min): $p=0.060$; and saline (0-100 min) versus CFA (0-100 min): $p=0.301$).

All in all, data from experiment IV showed that the **increase in accumbal DA release elicited by systemic ethanol administration (1.5 g/kg s.c.) is blunted by the presence of inflammatory pain**.

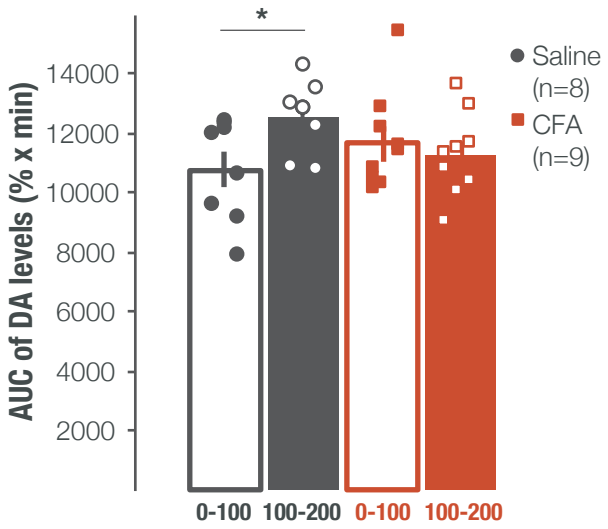

Figure 4.10 Global change in DA levels induced by saline (0 -100 min, empty bar) and ethanol (100-200 min, filled bar) calculated as AUC. Data are mean ± SEM in **black dots** for saline rats and in **red squares** for CFA rats *=p<0.05, significant differences (two-way ANOVA for repeated measures followed by Bonferroni multiple comparisons, p<0.05).

4.2.2 STUDY OF INFLAMMATORY PAIN IMPACT ON INTRA-VTA ETHANOL-INDUCED CPP

4.2.2.a Characterization of the CPP elicited by ethanol intra-VTA

Experiment V: Intra-VTA ethanol dose-response for CPP

Cannulae placement validation

The cannulae placements within the VTA were carefully confirmed by histological analysis. Figure 4.11 schematically depicts the location of the tip of the cannulae placements in the VTA for all the rats included in the present study. Only the data of the experiments in which the two cannulae were correctly placed were included in the statistical comparisons.

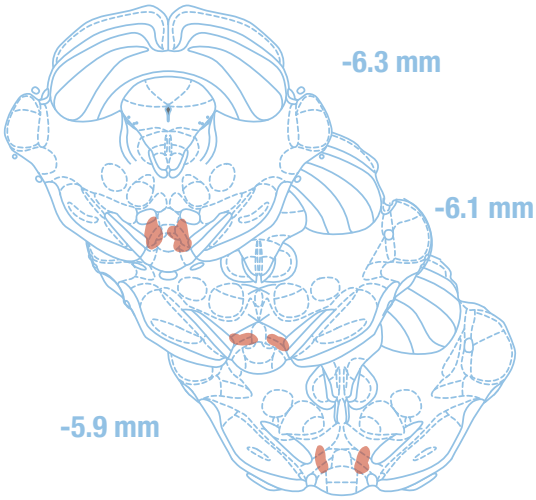

Figure 4.11 Schematic representation of the tip of the cannulae positions in animals belonging to experiment V. Due to the large number of animals it is represented, in orange, the area occupied by all the cannulae tips of the experimental groups: aCSF, 35 nmol, 70 nmol, 150 nmol, 300 mol and unpaired 70 nmol. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

Intra-VTA ethanol dose-response for CPP

The mean values of the preference score for each intra-VTA ethanol dose group are shown in Figure 4.12. These preference scores were calculated by subtracting the time spent in the drug-paired side during Pretest from the time spent in the drug-paired side during Test. Results showed that **ethanol significantly modifies the time spent in the drug-paired compartment during the test session compared with the pretest** (Brown-Forsythe test, $p<0.001$). Both, the group administered with **70 and 150 nmol** of ethanol showed a **significant increase in the preference score** for the drug-paired compartment as compared with the control group (aCSF) (Games-Howell adjustment for multiple comparisons, $p=0.006$ and $p=0.042$, respectively), indicating the expression of CPP. The animals receiving 70 nmol of ethanol showed the highest preference (200 ± 26 seconds vs 145 ± 20 seconds for the 150 nmol group). On the other hand, **the lowest dose administered (35 nmol) did not induce any preference**. Interestingly, the **highest dose (300 nmol) induced a significant decrease in the preference score relative to the control group** (Games-Howell adjustment for multiple comparisons, $p=0.023$), suggesting the expression of CPA for this dose. The mean score for this dose was also significantly lower compared with that observed for animals treated with 70 nmol dose (Games-Howell adjustment for multiple comparisons, $p<0.001$). Additionally, the preference score from the **unpaired group conditioned with the 70 nmol dose did not differ from the control group** (aCSF) (Games-Howell adjustment for multiple comparisons, $p=1.000$).

Altogether, results in experiment V showed that **either reinforcing (CPP) or aversive (CPA) ethanol properties are dependent on the dose directly administered into the VTA** (ranging here from 35 to 300nmol).

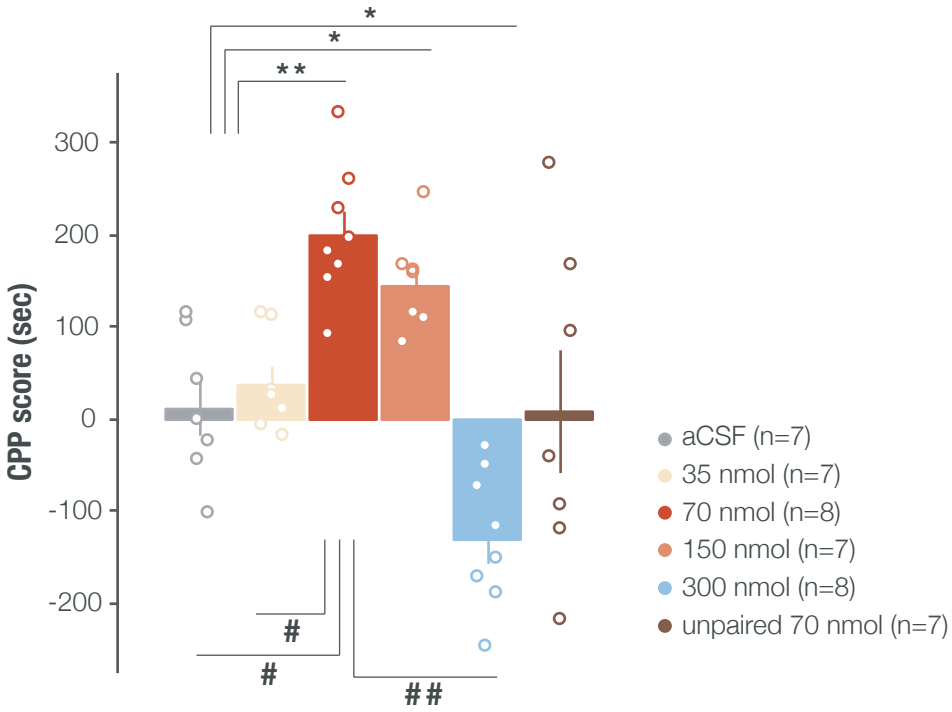

Figure 4.12 Dose-response relationship for the CPP elicited by the administration of intra-VTA ethanol. Data are mean ± SEM of the preference score (test minus pretest time spent in ethanol-paired compartment) in **grey** for aCSF, **beige** for 35 nmol, **red** for 70 nmol, **orange** for 150 nmol, **blue** for 300 mol and **brown** for unpaired 70 nmol treated animals. *=p<0.05, **=p<0.01, significant differences relative to the control group (aCSF); #=p<0.01, ##=p<0.001, significant differences relative to the 70 nmol treated group (Brown-Forsythe followed by Games-Howell test).

Expression of NR1 and NR2A NMDA receptor subunits after ethanol induced CPP or CPA

Western blot technique was selected to explore changes in NMDAR NR1 and NR2A subunits in the NAc and dorsal hippocampus and results are shown in Figures 4.13 and 4.14, respectively. The analysis was performed in brain tissue extracted from animals belonging to the groups that showed the highest preference or aversion (70 nmol and 300 nmol). We also analyze brains from animals belonging to both the unpaired and the control group.

The expression of **NR1 in the NAc did not significantly change** between groups (one-way ANOVA, $F_{(3,12)}$=2.050; p=0.161) although levels for the group conditioned with 70 nmol of ethanol were tend to be higher relative to controls (143 ± 12 %, expressed as percentage of change from controls). Also, this same group (70 nmol treated) showed the highest level of **NR2A** (166 ± 27 %, expressed as percentage of change from controls); however, the statistical analysis also showed **no significant differences between groups** (Kruskal-Wallis test, p=0.126).

In the **hippocampus**, the **level of expression of both NR1 and NR2A did not differ** among groups (one-way ANOVA, $F_{(3,11)}$=2.078; p=0.131 and $F_{(3,12)}$=1.066; p=0.403, respectively).

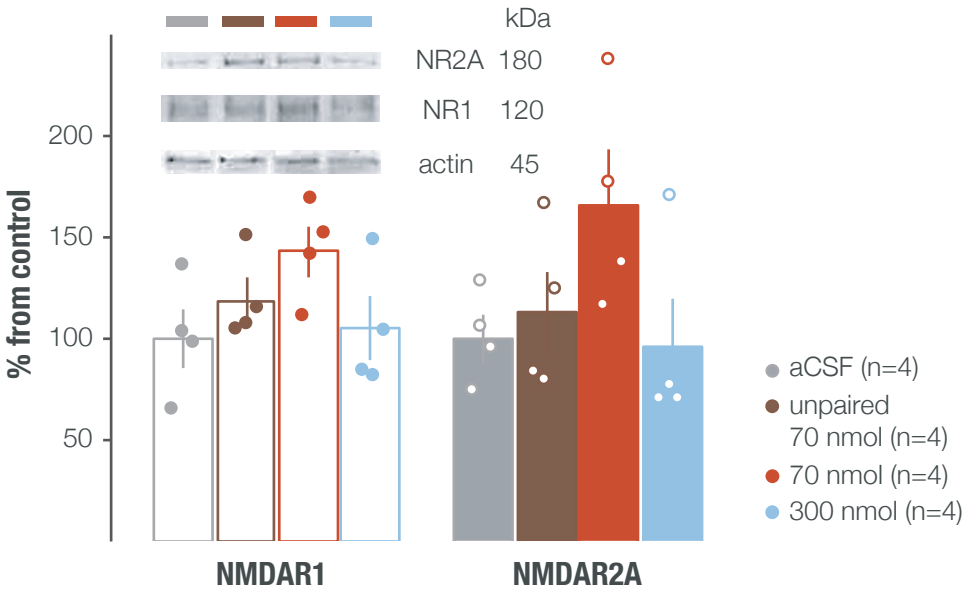

Figure 4.13 NMDA subunit expression in NAc after ethanol induced CPP or CPA. Data are represented as mean ± SEM of the percentage from control group in **grey** for aCSF, **brown** for unpaired 70 nmol, **red** for 70 nmol and **blue** for 300 nmol treated animals.

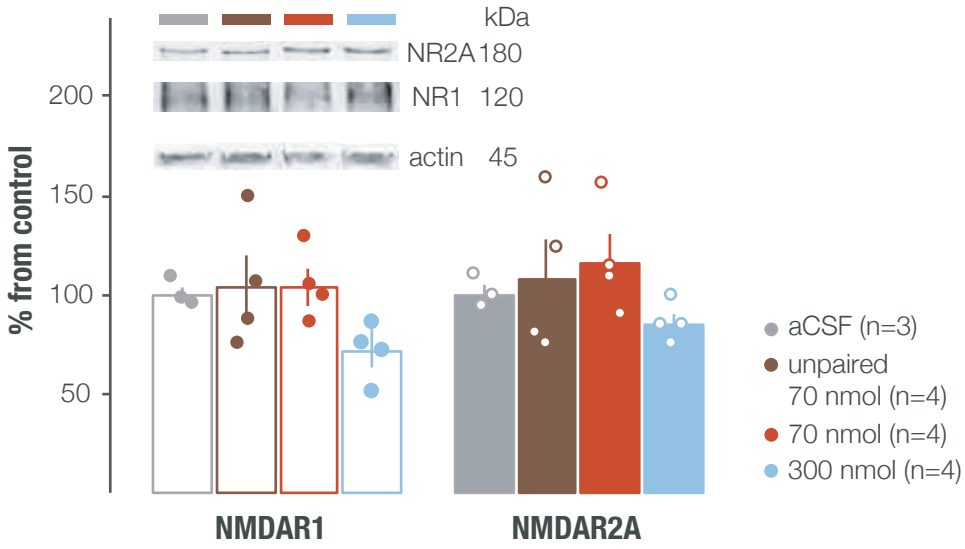

Figure 4.14 NMDA subunit expression in the hippocampus after ethanol induced CPP or CPA. Data are represented as mean ± SEM of the percentage from control group in **grey** for aCSF, **brown** for unpaired 70 nmol, **red** for 70 nmol and **blue** for 300 nmol treated animals.

To further analyze the tendency in the changes of NR2A expression, a different cohort of animals was conditioned with **70 nmol and aCSF** as control group. The objective was to **replicate the previous behavioral results** and perform a more precise analysis of the NR1 and NR2A mRNA expression through the use of the *in situ* hybridization technique. Again, only the data of the experiments in which the two cannulae were correctly placed were included in the statistical comparisons and a schematic representation for the location of the cannula tips in the VTA can be found in Figure 4.15.

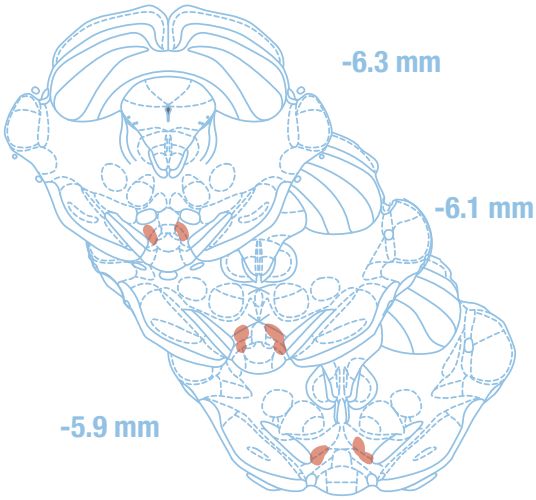

Figure 4.15 Schematic representation of the tip of the cannulae positions in animals belonging to the new cohort of experiment V. In orange, the area occupied by all the cannulae tips of the experimental groups: aCSF and 70 nmol. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

As shown in Figure 4.16 and in accordance with the previous data, **70 nmol of ethanol significantly increased the time spent in the drug-paired compartment** (p=0.009, t-test).

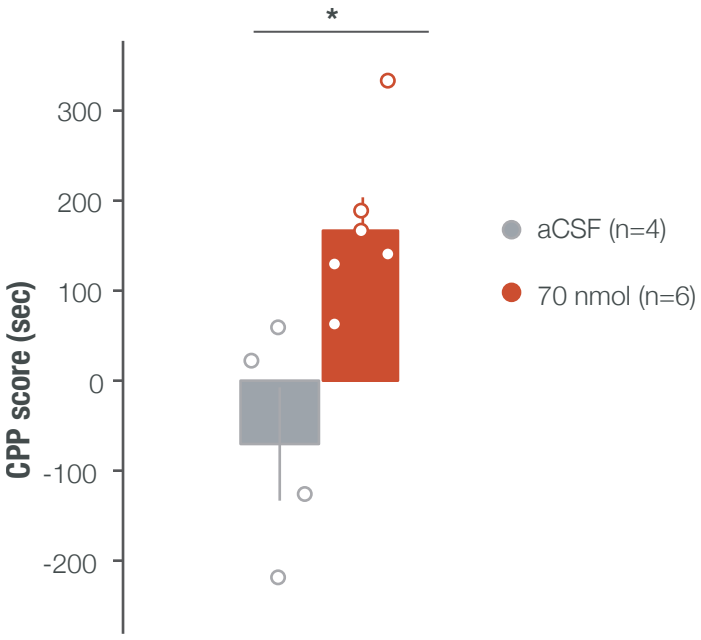

Figure 4.16 Place preference elicited by the administration of intra-VTA ethanol. Data are mean ± SEM of the preference score (test minus pretest time spent in ethanol-paired compartment) in **grey** for aCSF, and **red** for 70 nmol treated animals. *=p<0.01, t-test.

The *in situ* hybridization quantifications are represented in Figure 4.17 and 4.18 for the NAc and hippocampus, respectively. Also, representative pictures of the images obtained by the *in situ* RNAscope technique can be found in Figure 4.19, Figure 4.20 and Figure 4.21. The results revealed that, **in the NAc,** the group that **expressed CPP after ethanol treatment has significantly higher mRNA levels of NR2A** (5.64 ± 0.54 dots/cell) as compared with the control group (3.62 ± 0.03 dots/cell) (t-test, p=0.014). However, the t-test did **not reveal significant difference for NR1 mRNA** levels between the two groups (p=0.316). Besides, in the **hippocampus**, the levels of **NR1 and NR2A mRNA expression were not different** between ethanol conditioned and control animals, as shown by the t-test (p=0.896 and p=0.698).

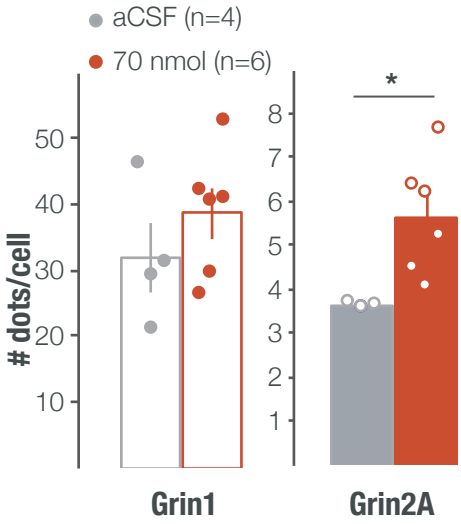

Figure 4.17 Expression of mRNA from NMDA subunits NR1 (Grin 1) and NR2A (Grin 2A) in the NAc after ethanol induced CPP. Data are mean ± SEM represented as number of dots (mRNA molecules) per cell in **grey** for aCSF, and **red** for 70 nmol treated animals. *=p<0.05, t-test.

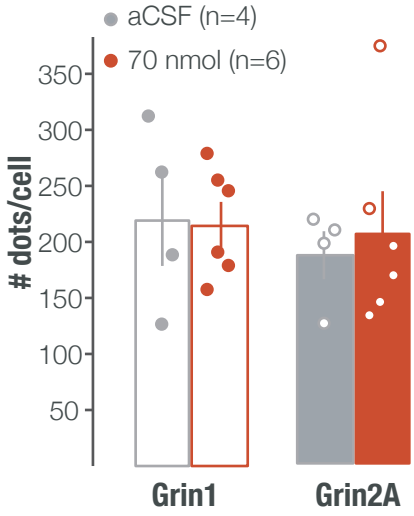

Figure 4.18 Expression of mRNA from NMDA subunits NR1 (Grin 1) and NR2A (Grin 2A) in the hippocampus after ethanol induced CPP. Data are mean ± SEM represented as number of dots (mRNA molecules) per cell in **grey** for aCSF, and **red** for 70 nmol treated animals. *=p<0.05, t-test.

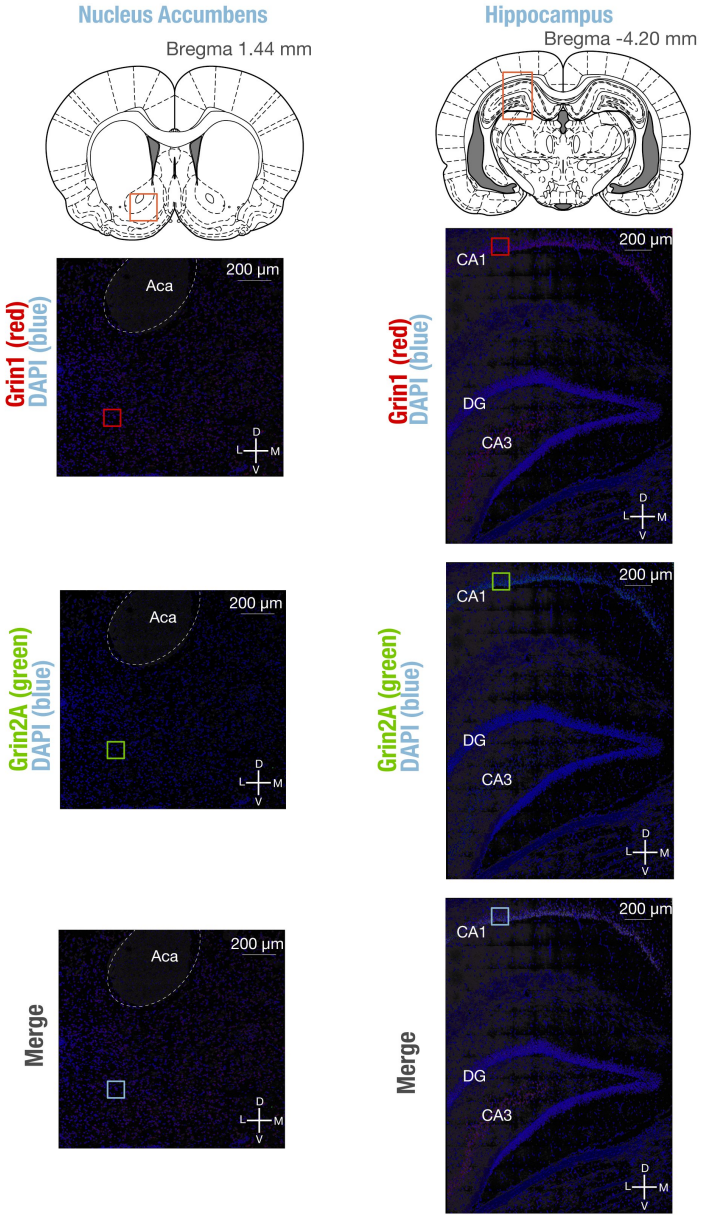

Figure 4.19 Representative pictures of the compilation files obtained in the mRNA *in situ* Hybridization Assay used for the quantification of mRNA from NR1 and NR2A subunits in NAc (left) and hippocampus (right) after ethanol induced CPP. Squares delimit the area of the amplified pictures shown in Figure 4.20 for the NAc and in Figure 4.21 for the hippocampus. Abbrevietions: Aca, anterior commissure; CA3, field CA3 of hippocampus; DG, dentate gyrus; CA1, field CA1 of hippocampus.

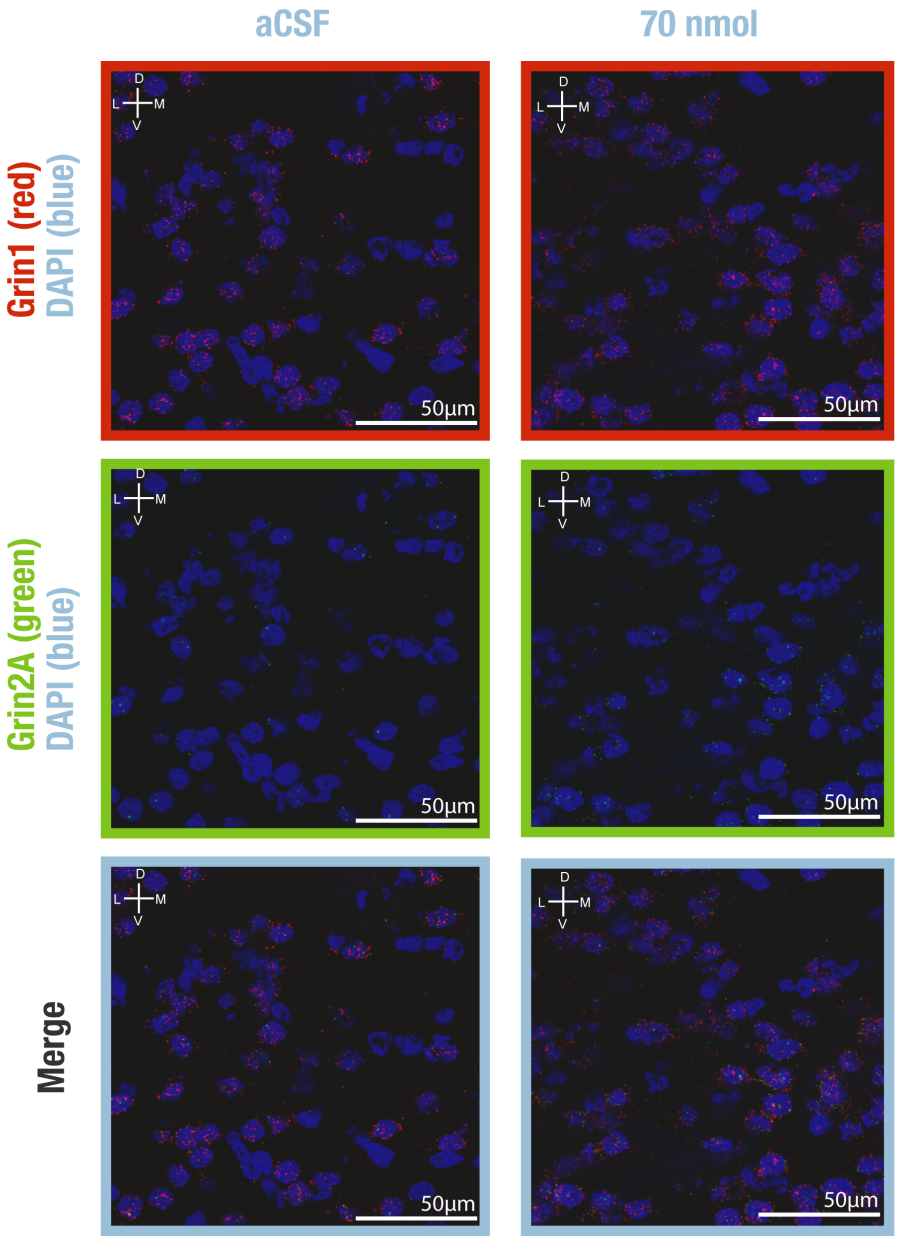

Figure 4.20 Representative amplified pictures obtained in the mRNA *in situ* Hybridization Assay used for the quantification of mRNA from NR1 and NR2A subunits in NAc. Amplified pictures from one animal of each group (aCSF on the left and ethanol 70 nmol on the right) were selected.

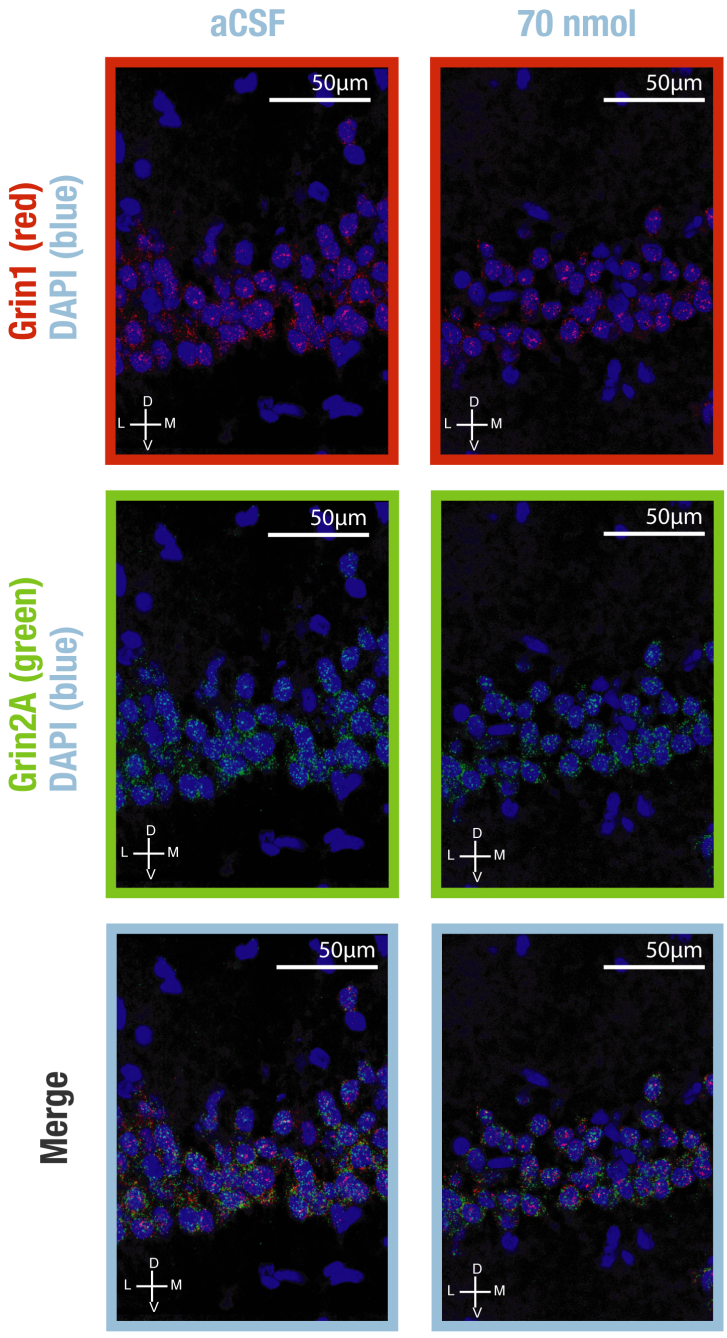

Figure 4.21 Representative amplified pictures obtained in the mRNA *in situ* Hybridization Assay used for the quantification of mRNA from NR1 and NR2A subunits in hippocampus. Amplified pictures from one animal of each group (aCSF on the left and ethanol 70 nmol on the right) were selected.

In whole, the analysis of NMDA expression in experiment V showed that the expression of **CPP induced by 70 nmol correlates with increased levels of mRNA from NR2A subunit in the NAc**, whereas **no changes** are observed in the **hippocampus**.

Experiment VI: MORs role in ethanol CPP acquisition

Cannulae placement validation

The cannulae placements within the VTA were carefully confirmed by histological analysis. Figure 4.21 schematically depicts the location of the tip of the cannulae in the VTA for all the rats included in the present study. Only the data from rats in which the two cannulae were correctly placed were included in the statistical comparisons.

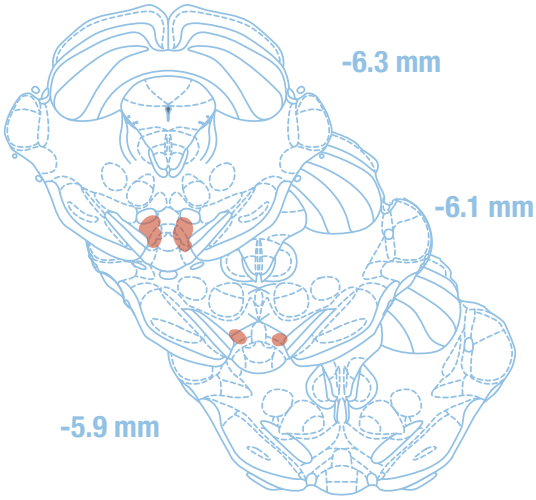

Figure 4.22 Schematic representation of the tip of the cannulae positions in animals belonging to experiment V. In orange, the area occupied by all the cannulae tips of the experimental groups: aCSF-aCSF, β-FNA+aCSF, aCFS+70nmol and β-FNA+70nmol. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

Effect of MORs blockade on ethanol CPP acquisition

The mean values of the preference score for each group, calculated by subtracting the time spent in the drug-paired side during Pretest from the time spent in the drug-paired side during Test, are shown in Figure 4.23. Results show that the **pretreatment with β-FNA was able to block the acquisition of the CPP induced by** the **ethanol** dose that previously produced the highest preference score (**70 nmol**). The statistical analysis confirmed that only the group pretreated with aCSF and conditioned with 70 nmol of ethanol presented a significantly higher score (204 ± 19 sec) compared to the control groups treated with aCSF and pretreated with aCSF (20 ± 29 sec) or with β-FNA (-2 ± 36 sec). Also, and more importantly, the score obtained for this group also significantly differed from that obtained in the group pretreated with β-FNA and receiving ethanol 70 mol (25 ± 38 sec) (Kruskal-Wallis, $p=0.002$; pairwise comparisons with Bonferroni adjustment, $p<0.005$).

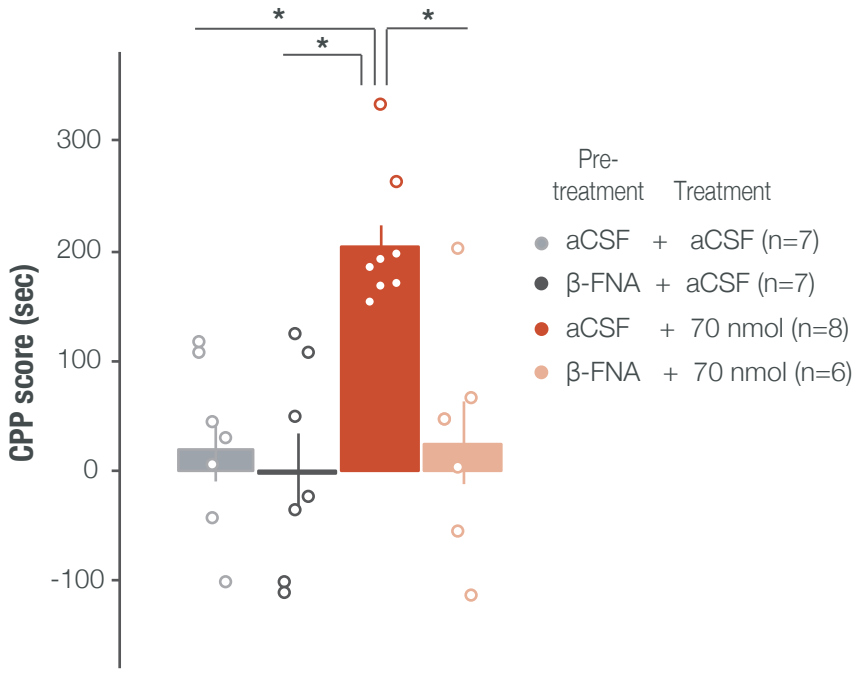

Figure 4.23 Effect of MORs blockade on ethanol CPP acquisition. Data are mean ± SEM represented as preference score (test minus pretest time spent in ethanol-paired compartment) in **light grey** for aCSF+aCFS, **dark grey** for β-FNA+aCSF, **red** for aCFS+70nmol and **light orange** for β-FNA+70nmol treated groups. *=p<0.05, significant differences relative to aCFS+70nmol group (Kruskal-Wallis followed by Bonferroni adjustment).

Besides, tissue samples from brains belonging to animals from the aCSF + aCSF and β-FNA + ethanol groups were analyzed for NR1 and NR2A subunits expression. Results that can be found in Figure 4.24. As can be seen, the **levels of NR1 and NR2A subunits were not significantly different** between groups (t-test, p=0.72 and non-parametric test p=0.670, respectively).

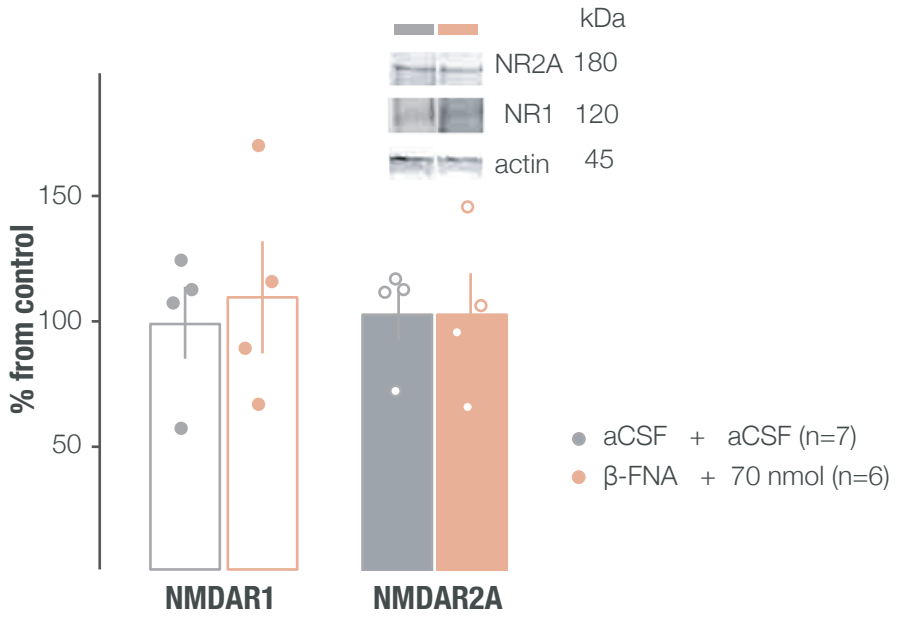

Figure 4.24 NMDA subunit expression in the NAc after blockade of ethanol CPP acquisition. Data are represented as mean ± SEM of the percentage from control group **light grey** for aCSF+aCFS and **light orange** for β-FNA+70nmol treated groups.

Overall, results from experiment VI showed that the **blockade of VTA MORs with β-FNA impairs the development of ethanol induced CPP at the dose of 70 nmol**. In addition, in this group there is no change in the expression of NMDA subunits in the NAc.

4.2.2.b Experiment VII: Effect of inflammatory pain on intra-VTA ethanol-induced CPP

Cannulae placement validation

The cannulae placements within the VTA were carefully confirmed by histological analysis. Figure 4.25 schematically depicts the location of the tip of the cannulae in the VTA for all the rats included in the present study. Only the data from animals in which the two cannulae were correctly positioned were included in the statistical comparisons.

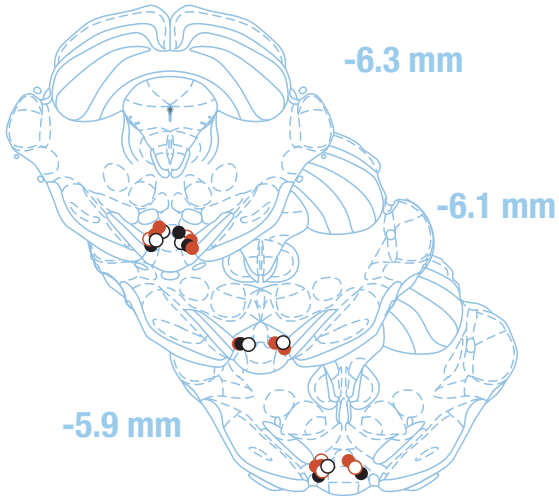

Figure 4.25 Schematic representation of the tip of the cannulae positions in animals belonging to experiment VII. Experimental groups are: aCSF (**blue**); saline-52nmol (**empty black**); saline-70nmol (**filled black**); CFA-52nmol (**empty red**); CFA-70nmol (**filled red**). Dots could represent more than one cannula placement. Numbers indicate distance from bregma. Adapted from Paxinos and Watson 2007.

Mechanical nociception evaluation

The Von Frey test was performed to measure the changes in mechanical nociception throughout the CPP experiment VII (see section 3.8.1) The results of the Von Frey test are represented in Figure 4.26 and confirmed that saline rats maintained the mechanical nociceptive threshold as the one measured in the baseline session. On the contrary, CFA rats showed a significant decrease in the mechanical nociceptive threshold that was maintained until the performance of the CPP Test session (two-way ANOVA for repeated measures, within-subjects interaction time x group $F_{(2,44)}=16.299$, $p<0.001$; Bonferroni correction for multiple comparisons, differences between groups: $p_{D2}<0.001$ and $p_{D9}<0.001$, differences from baseline, saline: $p_{D2}=1.000$ and $p_{D9}=1.000$, CFA: $p_{D2}<0.001$ and $p_{D9}<0.001$).

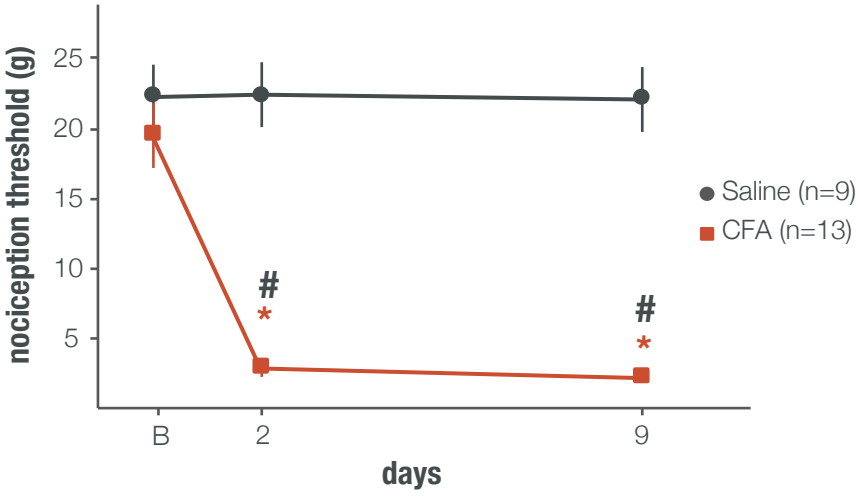

Figure 4.26 Von Frey test performed during experiment VII. Data are mean ± SEM and represent the paw withdrawal thresholds measurements in **black dots** for saline rats (n=9) and in **red squares** for CFA rats (n=13). *=p<0.001, significant differences between groups; #=p<0.001, significant differences relative to baseline (B) (two-way ANOVA for repeated measurements followed by Bonferroni multiple comparisons).

Effect of inflammatory pain on intra-VTA ethanol-induced CPP

In this experiment, we analyzed the ability of two different ethanol doses directly administered into the VTA to induce CPP in animals under pain and pain-free conditions. Results show that **inflammatory pain causes significant alterations on ethanol-induced CPP** (one-way ANOVA $F_{(4,23)}$=3.685, p=0.018, Figure 4.27). Concretely, **saline rats that received the lowest ethanol dose (52 nmol) showed a preference score significantly higher** than the control group (146 ± 26 vs -12 ± 36, LSD test, p=0.040), whereas **CFA rats did not developed a preference** for the ethanol paired compartment when compared to control group (-18 ± 49 vs -12 ± 36, LSD test, p=0.920). Interestingly, the **higher ethanol dose used (70 nmol) induced CPP in both saline and CFA rats**, shown as preference scores significantly higher than the control group (saline rats: 165 ± 54, p=0.016; CFA rats: 140 ± 44, LSD test, p=0.024). Moreover, the statistical analysis showed significant difference between doses only in CFA rats (saline rats: p=0.804, CFA rats: p=0.019, LSD test).

In summary, results from experiment VII showed that, in comparison with the saline group, **the ability of ethanol administered into the VTA to induce CPP is impaired in CFA animals the case of the lower dose of ethanol (52 nmol) but not when using a higher dose (70 nmol)**.

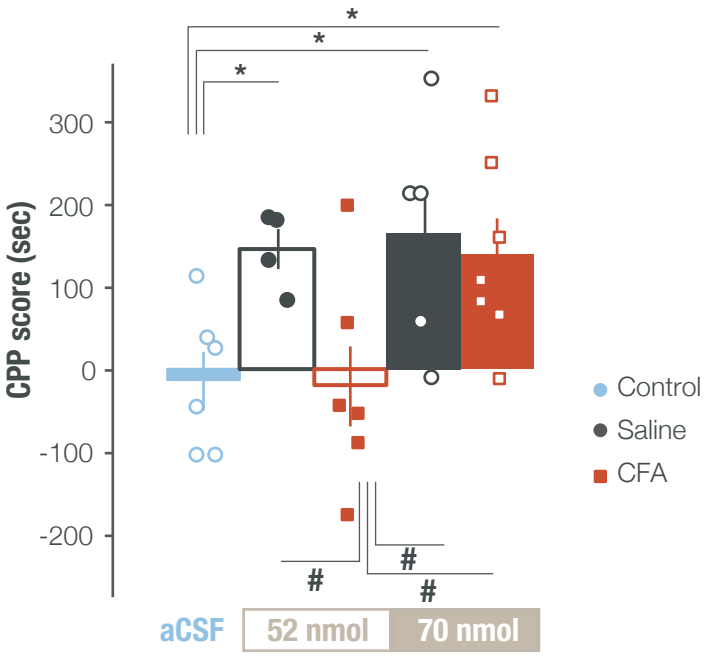

Figure 4.27 Effect of inflammatory pain on intra-VTA ethanol-induced CPP. Data are mean ± SEM and represent preference scores, calculated as test minus pretest time spent on the ethanol-paired compartment in **blue** for aCSF (n=6), **empty black** for saline-52nmol (n=4), **empty red** for CFA-52nmol (n=6), **filled black** for saline-70nmol (n=5) and **filled red** for CFA-70nmol (n=7) treated groups. *=p<0.05, significant differences relative to the control group (aCSF); #=p<0.05, significant differences relative to the CFA-52nmol group (one-way ANOVA followed by LSD test).

4.3 EFFECT OF INFLAMMATORY PAIN ON ETHANOL RELAPSE

As previously described in section 3.8.3, in **experiment VIII** the effect of inflammatory pain on ethanol relapse was studied by selecting a non-operant self-administration paradigm in which periods of continuous access to 4 different bottles (water and 5%, 10% and 20% (v/v) ethanol) were alternate with forced abstinence periods (access only to water). The saline or CFA injection was conducted forty-eight hours before the reintroduction of ethanol bottles after the last abstinence period.

During the whole experiment animals were regularly weighted to ensure their correct growth despite the long exposure to ethanol solutions. The **temporary evolution of weight** for the two groups can be found in Figure 4.28. As expected, **no significant differences** were found between the two groups **prior to the saline or CFA injection** (two-way ANOVA for repeated measures, within subjects effect of time $F_{(24,432)}=502.327$, $p<0.001$; interaction time x group $F_{(24,432)}=0.432$, $p=0.992$; between subjects effect of group $F_{(1,18)}=0.071$, $p=0.793$). Moreover, as can be seen in Figure 4.29, when analyzing rats weigh **during the last cycle** (e.g., prior and after to the saline or CFA injection) **no significant effects** are found between these two groups (two-way ANOVA for repeated measures, within subjects effect of time $F_{(3,54)}=112.224$, $p<0.001$; interaction time x group $F_{(3,54)}=1.869$, $p=0.146$; between subjects effect of group $F_{(1,18)}=0.019$, $p=0.891$).

Therefore, **nor the continuous ethanol intake neither the induction of an inflammatory pain condition** seemed to **alter rats weigh**t during experiment VIII.

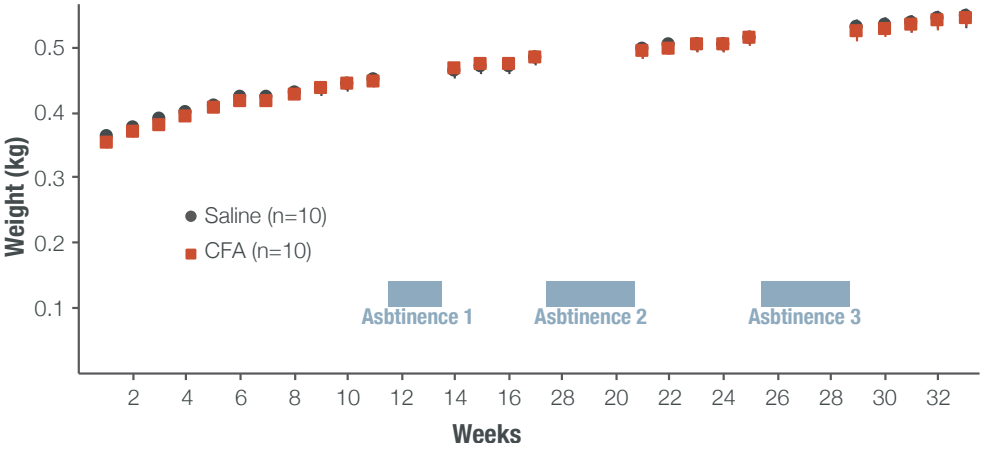

Figure 4.28 Weekly evolution of the rats weight prior to the 4th abstinence period. Data are mean ± SEM of animals weight (kg) shown in **black dots** for saline rats (n= 10) and in **red squares** for CFA rats (n=10).

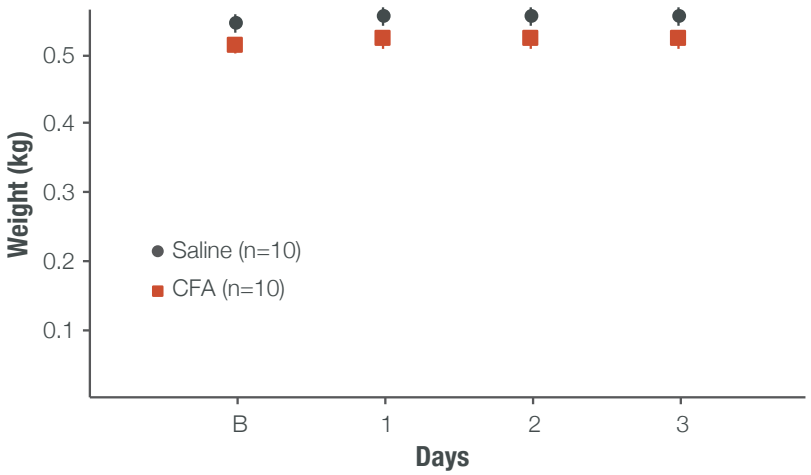

Figure 4.29 Mean of rats weight during the last three basal days (B) and for the three post-abstinence days after the last abstinence period. Data are mean ± SEM of animals weight (kg) shown in **black dots** for saline rats (n= 10) and in **red squares** for CFA rats (n=10).

Moreover, another interesting parameter that was monitored during the whole experiment VIII was the **total intake of liquid**, as all the bottles (water, 5%, 10% and 20% (v/v) ethanol) were regularly weighted. Again, **no significant differences were found between saline and CFA** selected rats during the whole timeframe **previous to the last abstinence period** (two-way ANOVA for repeated measures, within subjects effect of time $F_{(24,432)}=17.174$, $p<0.001$; interaction time x group $F_{(24,432)}=1.011$, $p=0.450$; between subjects effect of group $F_{(1,18)}=0.015$, $p=0.905$) (Figure 4.30). In the case of the **last abstinence period**, levels of **total intake of liquid during baseline and after ethanol reintroduction** (and, therefore, after saline or CFA injection), were **not significantly different** between saline and CFA rats (two-way ANOVA for repeated measures, within subjects effect of time $F_{(3,54)}=4.895$, $p<0.001$; interaction time x group $F_{(3,54)}=0.348$, $p=0791$; between subjects effect of group $F_{(1,18)}=0.590$, $p=0.453$) (Figure 4.31).

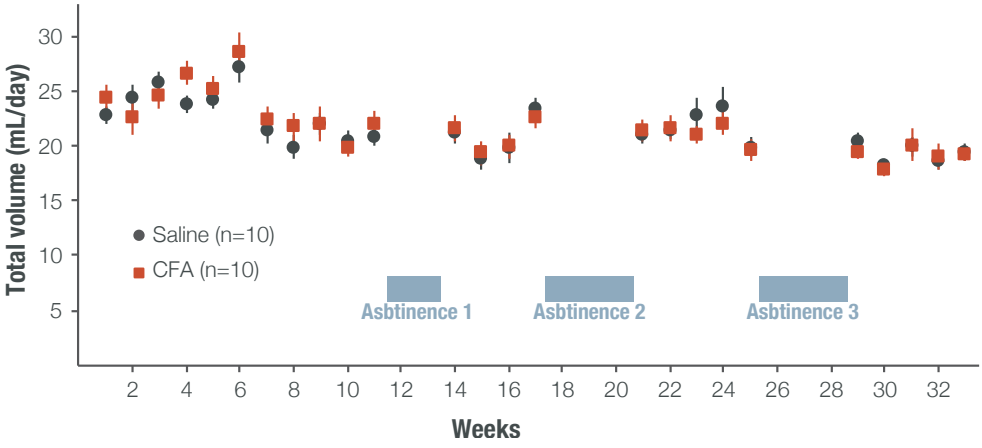

Figure 4.30 Weekly evolution of the rats total volume intake prior to the 4th abstinence period. Data are mean ± SEM of total volume (mL/day) shown in **black dots** for saline rats (n= 10) and in **red squares** for CFA rats (n=10).

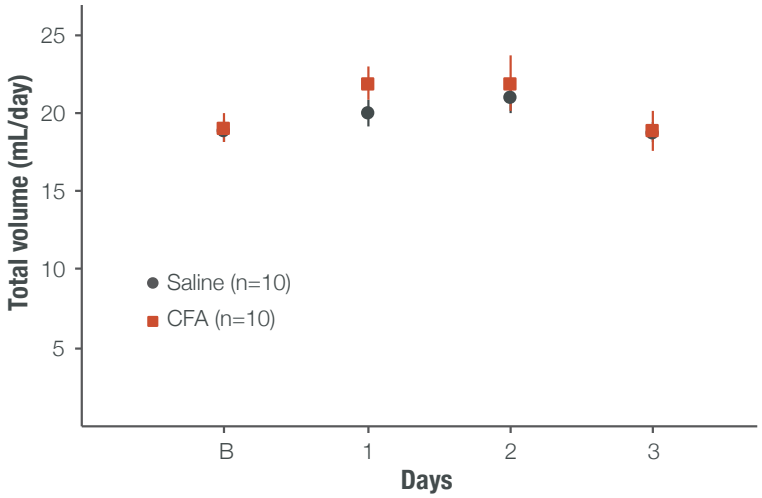

Figure 4.31 Mean of rats total volume intake during the last three basal days (B) and for the three post-abstinence days after the last abstinence period. Data are mean ± SEM of total volume (mL/day) shown in **black dots** for saline rats (n=10) and in **red squares** for CFA rats (n=10).

In addition, in Figure 4.32 it is represented the total amount of ethanol intake for each group along the whole ethanol exposure period previous to the last abstinence phase. As expected, **before the injection of saline or CFA**, there were **no significant differences in total ethanol intake** between the two experimental groups (two-way ANOVA for repeated measures, within subjects effect of time $F_{(24,432)}=4.361$, $p<0.001$; interaction time x group $F_{(24,432)}=0.562$, $p=0.955$; between subjects effect of group $F_{(1,18)}=1.589$, $p=0.224$).

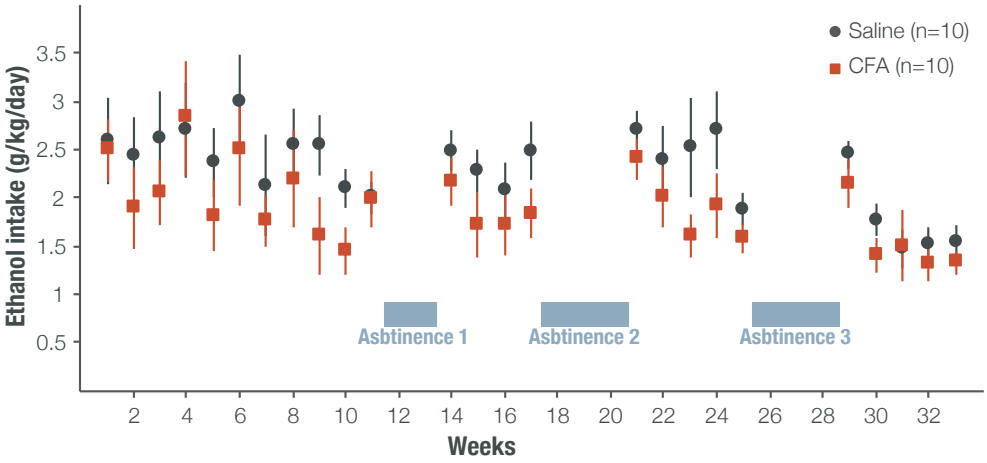

Figure 4.32 Weekly evolution of the rats total ethanol intake prior to the 4th abstinence period. Data are mean ± SEM of total ethanol intake (g/kg/day) shown in **black dots** for saline rats (n= 10) and in **red squares** for CFA rats (n=10).

For each cycle of exposure, basal and post-abstinence ethanol intake was measured as the mean of the three days before and after to the abstinence period, respectively. In the case of the three first cycles of exposure prior to the saline or CFA injection, **no significant differences were found between cycles** in both saline (two-way ANOVA for repeated measures, within subjects effect of period $F_{(1,27)}$=13.217, p=0.001; interaction period x cycle $F_{(2,27)}$=1.029, p=0.371; between subjects effect of cycle $F_{(2,27)}$=1.163, p=0.328) (Figure 4.33, left) and CFA groups (two-way ANOVA for repeated measures, within subjects effect of period $F_{(1,27)}$=41.348, p<0.001; interaction period x cycle $F_{(2,27)}$=1.338, p=0.281; between subjects effect of cycle $F_{(2,27)}$=0.663, p=0.523) (Figure 4.33, right). Furthermore, both s**aline and CFA selected rats showed an increase in total ethanol intake** after the first three abstinence periods (prior to the saline or CFA injection). This increase was **statistically significant in two of the three cycles** for saline and CFA selected rats (e.g., the ADE occurs after two of the three abstinence periods) and, also, **both groups**

showed an ADE after the third abstinence period, that was the last one before the saline or CFA injection (paired t-test, saline: $p_{1stcycle}=0.005$, $p_{2ndcycle}=0.401$, $p_{3rdcycle}=0.010$; CFA: $p_{1stcycle}=0.056$, $p_{2ndcycle}<0.001$, $p_{3rdcycle}=0.007$).

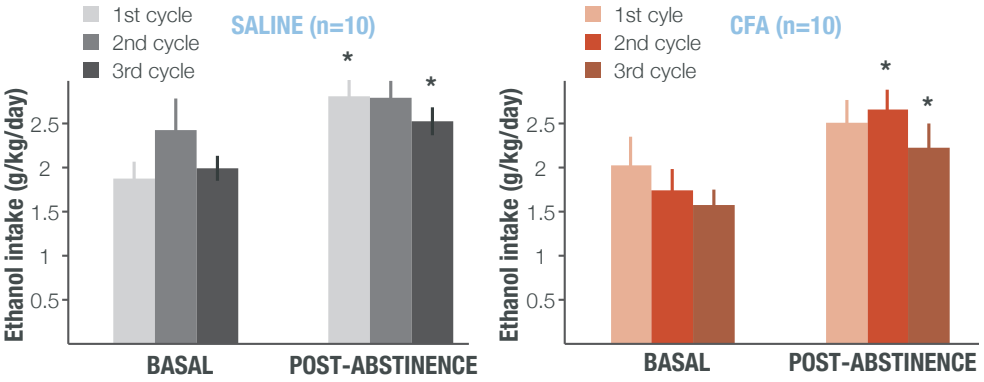

Figure 4.33 Total ethanol intake before and after the first three abstinence periods. Data are mean ± SEM of total alcohol intake (g/kg/day) of the 3 days pre- (basal) and post-abstinence shown in light grey (1st cycle), **grey** (2nd cycle) and **dark grey** for the saline selected rats (left, n=10) and in light orange (1st cycle), **red** (2nd cycle) and **dark red** (3rd cycle) for the CFA selected rats (right, n=10). *=p<0.05, significant differences between respective basal and post-abstinence consumptions (paired t-test).

During the **last abstinence period** and forty-eight hours before ethanol reintroduction, animals were injected with saline or CFA in the hindpaw. ADE was analyzed by measuring ethanol consumption during the three days after reintroduction and comparing them to ethanol consumption during the three days before the abstinence period (basal), as can be found in Figure 4.34. After the reintroduction of alcohol solutions, **both CFA and saline rats showed an increase in alcohol consumption**. **No significant differences were found between groups** (two-way ANOVA for repeated measures, within subjects effect of time $F_{(1,18)}=45.599$, $p<0.001$; interaction time x group $F_{(1,18)}=0.536$,

p=0.474; between subjects effect of group $F_{(1,18)}$=1.647, p=0.216). Bonferroni correction for multiple comparisons showed significant differences between basal and post-abstinence ethanol consumption for both groups ($p_{(saline)}$<0.001, $p_{(CFA)}$=0.001) indicating the occurrence of ADE.

The **total ethanol intake for each of the three post-abstinence days** was also analyzed (Figure 4.35). Statistical analysis revealed a significant increase in ethanol intake after ethanol reintroduction both in CFA and saline rats, but there were no significant differences in intake between groups (two-way ANOVA for repeated measures, within subjects effect of time $F_{(3,54)}$=15.268, p<0.001; interaction time x group $F_{(3,54)}$=0.306, p=0.821; between subjects effect of group $F_{(1,18)}$=0.206, p=0.087). Concretely, in saline rats, total ethanol intake was significantly higher the 3 post-abstinence days compared to baseline (Bonferroni correction for multiple comparisons, $p_{(day1)}$=0.001, $p_{(day2)}$=0.019, $p_{(day3)}$=0.026), whereas in CFA rats there were only significant differences in total ethanol intake in the post-abstinence day 1 compared to that observed in baseline (Bonferroni correction for multiple comparisons, $p_{(day1)}$=0.001, $p_{(day2)}$=0.061, $p_{(day3)}$=1.000).

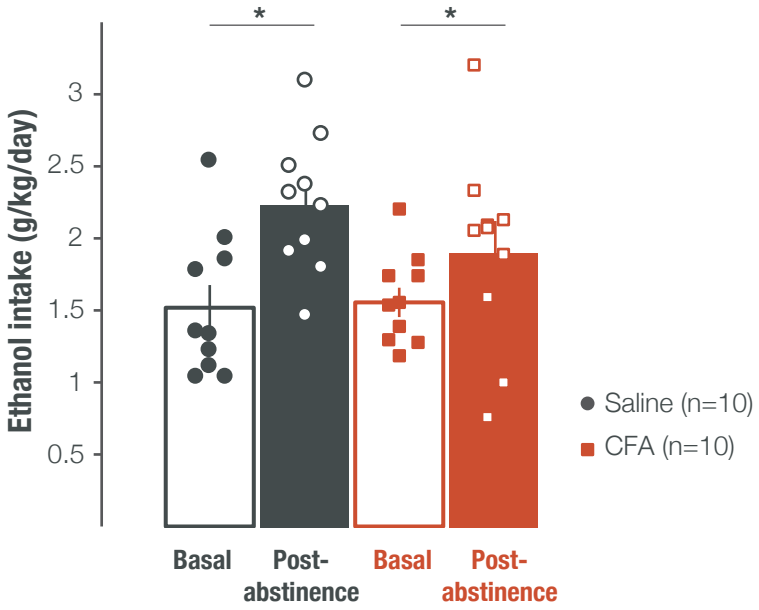

Figure 4.34 Total ethanol intake before and after the last abstinence period. Data are mean ± SEM of total alcohol intake (g/kg/day) of the 3 days pre- (basal, empty bar) and post-abstinence (filled bars) shown in **black** for saline rats (n= 10) and in **red** for CFA rats (n=10). *=p<0.001, significant differences between respective basal and post-abstinence consumptions (two-way ANOVA for repeated measures followed by Bonferroni correction for multiple comparisons).

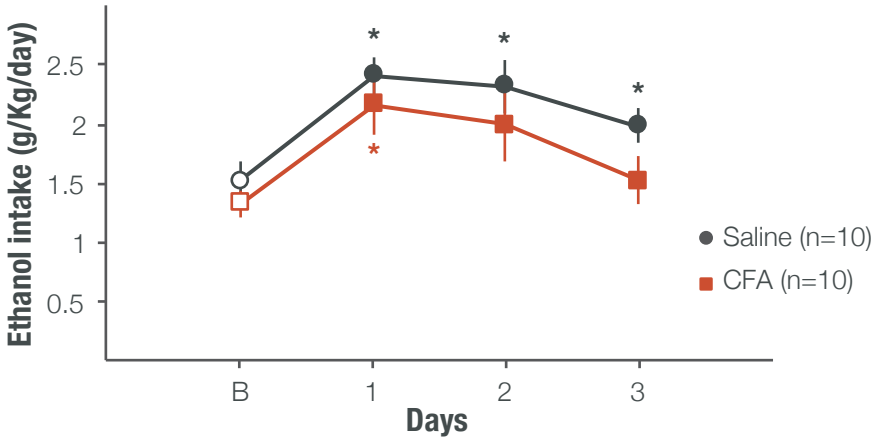

Figure 4.35 Mean of basal intake and post-abstinence ethanol intake per day after the last abstinence period. Data are mean ± SEM of total alcohol intake (g/kg/day) for basal (empty) and for each of the three post-abstinence days (filled) shown in **black dots** for saline rats (n= 10) and in **red squares** for CFA rats (n=10). *=p<0.05, significant differences relative to basal (two-way ANOVA for repeated measures followed by Bonferroni correction for multiple comparisons).

5 DISCUSSION

Globally, the results presented in this Thesis highlight the effects that inflammatory pain induces over the regulatory control that the EOS exerts on MCLS and, consequently, the impact of inflammatory pain in the ethanol reinforcing properties. In this way, inflammatory pain induces a **desensitization of local MORs in the VTA**, which results in changes in the ethanol actions on the activity of DA neurons in the MCLS. Concretely, inflammatory pain **reduces ethanol-evoked DA release in the NAc**. One important behavioral consequence is that pain **blunts the CPP** elicited by direct **ethanol** administration into the VTA. Finally, in the long-term ethanol exposure paradigm employed to evaluate the influence of inflammatory pain on relapse, the **ADE is manifested** in pain suffering animals, although **no changes** are observed **in its magnitude** compared to control animals.

Given the results obtained in the present Thesis, **three main questions** can be derived, which will be the focus to discuss in the following section: (1) Is the function of MORs in the MCLS altered by the presence of inflammatory pain? (2) Does inflammatory pain alter the ethanol-induced actions in the MCLS? (3) How does inflammatory pain impact on the ADE in long-term experienced animals?

5.1 IS THE FUNCTION OF MORS IN THE MCLS ALTERED BY THE PRESENCE OF INFLAMMATORY PAIN?

MORs are essential for reward processing of both natural stimuli and drugs of abuse (reviewed in Le Merrer 2009). The activation of MORs located in the VTA increases DA release in its projecting regions, a mechanism that has been traditionally implicated in the reinforcing properties of drugs, including ethanol (Charbogne et al., 2014, Darcq and Kieffer 2018; Hipólito et al., 2009; Kieffer and Gavériaux-Ruff 2002; Martí-Prats et al., 2015; Melis et al., 2009; Xiao and Ye 2008; Xie et al., 2012).

In addition, previous studies suggest that **pain induces plastic changes in the MCLS** (section 1.3.2.c) (Navratilova and Porreca 2014; Taylor 2017). Precisely, several studies analyzing this connection have revealed that subjects under persistent pain show an increase in the functional cortico-striatal connectivity in humans (Baliki et al., 2012) and a reduced phasic DA singling in both humans (Loggia et al., 2014) and rodents (Ozaki et al., 2002; Ren et al., 2015; Taylor et al., 2015). Also, persistent pain has been showed to modify motivational behavior for drugs (especially studied in opioids) (Massaly et al., 2016; Martin et al., 2007) and for natural rewards (Massaly et al., 2019; Schwartz et al., 2014). A possible mechanism contributing to these events could be a decrease in MOR function in the MCLS. In fact, a decrease in MOR function in many other regions involved in pain processing has been widely reported (see section 1.3.2.b) (Aoki et al., 2014; Hurley and Hammond 2001; Jongeling et al., 2009; Shaqura et al., 2004; Zhang et al., 2004). Therefore, it could be possible that local MOR function in the VTA is also

decreased by the presence of pain and, consequently, changes in the regulation of VTA DA neurons would take place.

For all that, the first group of experiments in this Thesis analyzed the **effect of inflammatory pain on the responsiveness of VTA MORs to acute administration of a pure agonist**. With this purpose, the expression of **cFos** was selected as an indirect measure of cell activation. The detection of this protein has been frequently used since the late 1980 (Bullitt 1990; Dragunow and Faull 1989) for mapping groups of neurons that display changes in their activity. Changes in neuronal activity lead to second messenger signaling cascades that induce the expression of the immediate early gene *c-fos,* which induces the production of the transcription factor cFos (Curran et al., 1984; Herdegen and Leah 1998). Therefore, in the first group of experiments, cFos expression was chosen to map the activation of VTA projecting areas after local MOR stimulation. This technique was considered the most appropriate because: i) it is a rapid and validated method to indirectly measure neuronal activation ii) cFos expression occurs after an acute activation such as intra VTA MOR agonist infusion and iii) this method allows to simultaneously analyze different brain regions.

Results from experiment I showed that the **cFos expression in projecting areas of the VTA after intra-VTA administration of DAMGO (an agonist of MORs) is reduced or suppressed in the majority of regions by the presence of inflammatory pain**. In control rats (no pain condition), (**experiment Ia**), local MOR agonism in the VTA increases cFos expression in the majority of its projection areas (NAc, BLA, ACC and BNST), with the exception of PL, IL and VP. However, when rats were under an inflammatory

pain condition (CFA rats, **experiment Ib**), this response was significantly diminished in the majority of regions (BLA, ACC and BNST).

Results obtained in **experiment Ia** also reveal the complexity of the opioidergic control of the activity of VTA projections (Table 4.1). For example, in the case of **NAc** and **BLA**, the administration of **DAMGO 14 ng**, but not DAMGO 7 ng, **significantly increased cFos-IR** counting as compared to aCSF administration. This increase may be due to a higher proportion of VTA DA neurons in the projections from the VTA to the NAc and BLA (Juarez and Han 2016; Lammel et al., 2014). Conversely, in the **ACC**, **cFos-IR counting was increased** when administering **DAMGO 7 ng** in the VTA but not when DAMGO 14 ng was injected. By contrast, neither PL nor IL were significantly activated by either dose. These observations are in agreement with previous data showing that the activation of MORs in the VTA increases DA release in the ACC but not in other prefrontal areas (Narita et al., 2010). Similarly to ACC, the **low dose of DAMGO increased cFos-IR** counts in the **BNST**. Although they have received less attention, there is also evidence that the BNST receives dopaminergic input from the VTA (Badiani et al., 2011), that could be activated by focal injections of the MOR agonist. However, the lack of effect of the higher dose of DAMGO in both ACC and BNST suggests that the opioidergic control of the VTA over these projecting regions is certainly complex. In this sense, the inhibitory GABAergic projections that the VTA also sends onto the PFC (Carr and Sesack 2000), might counteract the activation induced by dopaminergic projections, adding complexity to the opioidergic modulation of VTA efferences. Moreover, the DA effect on the PFC neuronal activity has been shown to have an inverted U-shape dose-response curve, as shown by electrophysiological recordings of PFC and VTA co-cultured cells. (Kroener et al., 2009). Therefore, even if DAMGO 14 ng induces higher levels

of DA in the ACC, the resulting effect could be a reduction of the cFos-IR cells. Finally, in the case of the **VP**, the activation of MORs in the VTA does **not increase cFos expression**. Although a possible lack of effect of the employed doses of DAMGO should not be discarded, our results suggest that the VTA projections to the VP could not be under MORs control (Lammel et al., 2014; Yoo et al., 2016).

Very interestingly, results from **experiment Ib** show that the presence of **inflammatory pain impaired the above-described increase of cFos expression induced by intra-VTA DAMGO** in all areas analyzed, except in NAc (Table 4.2). Previous studies have already shown that pain-induced desensitization of VTA MORs has an effect on the NAc activity (Ozaki et al., 2002). Concretely, in these studies authors showed that neuropathic pain decreases the opioid receptor-mediated G-protein activation induced by local MOR agonist in the VTA. Furthermore, the enhancement of DA release in the NAc stimulated by systemic morphine administration was significantly suppressed by sciatic nerve ligation. Surprisingly, in the present study, **cFos-IR in the NAc was higher after the administration of 14 ng of DAMGO in both saline and CFA rats**. Given the previously mentioned findings, a more expected result would have been to find that the dose that significantly increases cFos-IR counting in the NAc of control animals (14 ng of DAMGO) does not induce such an increase in CFA rats. This would have supported the fact that local MORs desensitization in the VTA has consequences in opioid induced activation of DA neurons projecting to the NAc. **Several reasons may prevent to observe a shift in the dose response curve in this experiment**. First of all, we may notice that it was not possible to detect an effect for the lower dose of DAMGO in the experiment Ia (saline rats). As commented above, in saline rats, the administration of DAMGO 7 ng elicited an increase

of cFos-IR in the NAc very similar to the one elicited by the DAMGO 14 ng dose. In fact, when compared to the aCSF counting, the lower DAMGO dose (7 ng) increased cFos-IR up to 169% in saline rats whereas this increase was not so noteworthy in CFA rats (145%) Nevertheless, these increases were not statistically significant when compared to aCSF counting in neither of the two experimental conditions (saline nor CFA). Second, it is possible that 14 ng is already a high dose that activates the dense projections from VTA to NAc, overcoming the lack of MORs effect. In this case, it is possible that an intermediate DAMGO dose would be useful to detect an increase of cFos expression only in saline treated animals. And third, it is important to note that cFos expression is the result of several events occurring during a long period of time, not only a direct consequence of the increase in DA release. Thus, the current result may show that, in CFA rats, 14 ng of DAMGO administered intra-VTA can induce similar overall activation than in saline rats, although behavioral outputs could radically differ.

Contrary, in the other regions studied, inflammatory pain altered the pattern of cFos-IR observed in experiment Ia after the administration of 7 or 14 ng of DAMGO. In fact, **in the presence of inflammatory pain, intra-VTA DAMGO did not induce an activation of the BLA, ACC and BNST**. Therefore, the DAMGO doses that induce cFos expression in the aforementioned projecting regions in experiment Ia are insufficient to induce cFos expression in pain rats.

In the light of these previous results, **experiment II** and **experiment III** were designed with the aim of further exploring the effect of inflammatory pain on the **modulatory function of local MORs (both in the NAc and the VTA), by measuring accumbal DA release**. DA release in the VTA-NAc pathway

mediates the reinforcing properties of drugs and natural reinforcers and this DA release is tightly regulated by MORs in this pathway (see section 1.2.1) (Devine et al., 1993; Johnson and North 1992; Le Merrer et al., 2009; Olszewski et al., 2011; Wassum et al., 2009; Wise et al., 1995). Although results in experiment Ib did not show significant changes in cFos expression in NAc in pain-suffering rats after VTA MORs activation, there was, as mentioned above, bibliographic evidence suggesting that pain alters MORs function in the MCLS thus blunting MORs-evoked accumbal activation (Ozaki et al., 2002). Therefore, despite not observing pain-induced changes on MORs activation of this circuit by cFos expression measure (experiment I, results in NAc), it would be still possible to find differences in DA release evoked by agonists of MORs. In this way, results from **experiment III** revealed a **reduced ability of DAMGO (7 ng in the VTA) to increase DA release in the NAc of pain-suffering rats**, suggesting a decrease in MOR function (Figure 4.7, top panels). These findings are in accordance with previous researches in other laboratories that have reported a pain-induced impairment of mesolimbic DA activity. Concretely, imaging studies in humans with chronic pain have found lowered responsiveness within the DA MCLS in response to reinforcing stimuli when compared to healthy subjects (Loggia et al., 2014; Taylor et al., 2016). Also, animal studies corroborate this fact by reporting decreases in opioid-evoked DA release in pain suffering rodents (Ozaki et al., 2002; Taylor et al., 2015).

Very interestingly, the **intra-VTA administration of a close to 10× higher dose of DAMGO (67ng) significantly increased accumbal DA release in both saline and CFA rats** (Figure 4.7, bottom panels). This finding points to a possible **shift in the dose-response curve effect of DAMGO**, what further supports the fact that CFA-treated rats show a decreased function of

VTA MORs. Actually, several studies have described a pain-induced decrease of MORs function in brain regions involved in nociception (Aoki et al., 2014; Hurley and Hammond 2001; Jongeling et al., 2009). In addition, Ozaki et al., 2002, by combining the microdialysis and GTPγS techniques, concluded that, in the presence of neuropathic pain, rats showed a loss of morphine-induced DA release in the NAc due to reduced MORs function in the VTA. Therefore, higher doses of agonist were needed to overcome the pain-induced desensitization of MORs in the VTA and to elicit similar increases in DA release as in control animals (as we observe in our experiment III). Also in vivo electrophysiological studies have shown that inflammatory pain alters MOR-mediated GABA transmission in the VTA (Hipólito et al., 2015). Concretely, in neurons from CFA-treated rats, the lower DAMGO dose was unable to decrease GABA release in the VTA, whereas a higher dose was able to overcome that MOR desensitization.

On the other hand, results obtained in **experiment II** showed that **NAc DA release evoked by the intra-NAc administration of DAMGO 10μM did not show significant differences between saline and CFA groups** (Figure 4.5). These results would suggest that **pain-induced loss of MOR-mediated modulation of DA** transmission was **mainly localized in the VTA**, and not in the NAc. However, it is important to notice that different results can be found when analyzing the individual effect of DAMGO 10μM on accumbal DA levels in each experimental group (saline or CFA-treated rats). In this way, in the saline group, the perfusion of DAMGO 10μM into the NAc was able to increase DA levels comparing to baseline. But, interestingly, the statistical analysis did not revealed an increase in DA levels in the CFA group after this same treatment. Thus, it could be that the effect of pain on local MORs regulation of DA levels in the NAc is not as robust as in the case of VTA

MORs. Finally, it is also possible that the dose selected in experiment II is not the appropriate to observe differences between groups. In this case, it is likely that, if using a lower DAMGO dose, significant difference between groups would be manifested, in a similar manner as in experiment III.

Altogether, these results demonstrate that **VTA MORs are desensitized by the presence of inflammatory pain and, thus, the regulation of VTA DA neurons innervating different projecting areas is altered in pain suffering animals**. Those findings set up the point for the next experiments to deeply examine whether this pain-induced desensitization is somehow altering ethanol action on the MCLS.

5.2 DOES INFLAMMATORY PAIN ALTER THE ETHANOL-INDUCED ACTIONS IN THE MCLS?

There is evidence that ethanol reinforcing properties are, at least in part, mediated by MORs in the MCLS (Guan and Ye 2010; Sánchez-Catalán et al., 2009 Xiao et al., 2007; Xiao and Ye 2008). This fact, together with the previous findings that demonstrate a loss of VTA MORs function in CFA rats, gave rise to the second concrete objective of this Thesis. In this way, results from **experiment IV** and **experiment VII** revealed that both **ethanol-evoked DA release in the NAc and ethanol-induced place preference**, respectively, are **reduced by the presence of inflammatory pain**. In addition, an initial study previous to experiment VII was performed to characterize the behavioral paradigm, as it was the first ethanol-induced CPP performed with the local administration of this drug into the VTA. Concretely, **experiment V** and **experiment VI** were carried out and they evidenced, not only a **dose-response relationship for the CPP elicited by intra-VTA ethanol**, but also the **critical role of MORs** in this behavior, as well as the **involvement of the NR2A NMDAR subunit**.

5.2.1 EFFECT ON ETHANOL-EVOKED ACCUMBAL DA RELEASE

In **experiment IV**, the systemic administration of **ethanol did not provoke a significant increase in NAc DA levels in CFA rats** whereas a clear DA release increase was observed in the case of saline-treated rats (Figures 4.9 and 4.10). This finding further supports the fact that inflammatory pain alters the neurochemical response of the MCLS elicited by ethanol. Moreover, this fact would be a direct consequence of the previous results showing a pain-

induced loss of MOR function in the VTA, considering that this receptor has an important role in ethanol reinforcing properties (Guan and Ye 2010; Sánchez-Catalán et al., 2009 Xiao et al., 2007; Xiao and Ye 2008). In fact, previous researches similarly showed a decrease in opioid-evoked DA release in the NAc in rats under pain condition (Hipólito et al., 2015; Ozaki et al., 2002). Concretely, by using the same inflammatory pain model as the one selected in the present work, Hipólito and collaborators showed that the i.v. heroin-evoked DA release in the NAc is blunted in CFA rats (Hipólito et al., 2015). Curiously, the heroin-evoked increases of DA release described by Hipólito and collaborators started 15 min after heroin administration and differences between pain-free and CFA-treated groups were found 30 and 45 min after this administration. According to the present results, ethanol-evoked increases and differences between groups appear later on time. It is important to highlight, that acute stressful stimuli also increase DA levels in the NAc (Abercrombie et al., 1989). For that reason, in this experiment, rats received a previous saline s.c. injection with the objective of discriminating this stress-induced effect. Although DA levels after this prior injection were higher than baseline in both saline and CFA rats, that increase was not statistically significant for none of the groups. In addition, the **global effect elicited by ethanol in saline rats was significantly higher than the saline-induced total effect** (Figure 4.10). On the contrary, **saline and ethanol-induced total effect were not statistically different in CFA rats** (Figure 4.10), confirming that the current results are derived from the drug pharmacological properties and not by the injection procedure itself. After all, these microdialysis results clearly show that inflammatory pain blocks ethanol-evoked DA release in the NAc, which may have an effect on the reinforcing properties of this drug.

5.2.2 EFFECT ON ETHANOL-INDUCED CPP

5.2.2.a Characterization of the CPP elicited by ethanol intra-VTA

It is classically accepted that drug-reward related behaviors (such as CPP) (Spyraki et al., 1983; Young et al., 2014) are mediated by DA transmission within the NAc. Therefore, the **CPP was the selected paradigm** in the present Thesis, with the objective to show **if altered neurochemical function in the MCLS could be translated into abnormal changes in this behavior**.

CPP paradigm has been previously used to study pain-induced alterations on opioid mesolimbic activation (Narita et al., 2005; Ozaki et al., 2002). Nonetheless, when the drug administered is ethanol, the existing literature yields paradoxical CPP results. In fact, previous studies in rats have reported both CPP, CPA or no changes in the preference when ethanol is administered by **systemic routes** (Bahi and Dreyer 2012; Becker et al., 2006; Peana et al., 2008; Zarrindast et al., 2010). Furthermore, Walker and Ettenberg showed that **intracerebroventricular (icv)** ethanol administration was able to induce CPP at the dose of 180 nmol, whereas 120 or 240 nmol did not induce changes in the preference (Walker and Ettenberg 2007). However, **in none of these or other previous studies using the CPP paradigm ethanol has been administered directly into discrete regions of the MCLS.**

In addition, the development and expression of drug-context learning associations has also been classically related to glutamatergic transmission and glutamatergic dependent forms of neural plasticity induced by drugs (Hearing et al., 2018). As explained in section 1.3.3, **NMDARs play an important role in the development of contextual driven behaviors** for both natural rewards (Brigman et al., 2013; Dang et al., 2006; Parker et al., 2011; Yin et al., 2008)

and drugs (Beutler et al., 2011; Daglish et al., 2001; Ersche et al., 2006; Hearing et al., 2017; Heusner and Palmiter 2005; Prosser et al., 2006). Nonetheless, very few studies have focused on the concrete role of NMDARs subunits that mediate ethanol evoked CPP.

Derived from the lack of CPP studies analyzing the dose-response effect of local ethanol administration into the VTA, it was necessary to design an **initial set of experiments prior to the introduction of the inflammatory pain variable**. Although alternative routes of administration could have also been explored, the intra-VTA was the selected one, because the aforementioned results from this Thesis and other related works have already shown a pain-induced desensitization of local MORs in the VTA (see section 5.2.1). For all that, these experiments were planned with the objective of (i) demonstrating whether local administration of ethanol into the VTA is able to induce CPP in rats (ii) finding the appropriate intra-VTA ethanol dose that elicits CPP in a robust manner, (iii) exploring the implication of MORs in the CPP induced by intra-VTA ethanol and (iv) analyzing the changes in NMDARs subunits expression in ethanol conditioned animals.

In this way, results from **experiment V** show, for the first time, that the **intra-VTA** administration of **70 and 150 nmol doses of ethanol** paired with a specific context, results in a **preference for that drug-associated environment** (Figure 4.12). As explained in section 3.8.1, the score measurement was selected as the analysis method in these experiments. The reason for this decision was that the score is a within-subject measure, allowing to compare the different ethanol doses with a control (aCFS) group. In the very extensive review on CPP methods by Tzschentke (Tzschentke 2007), the author stated that ethanol preference or aversion results were equal across 4 different methods

for analyzing the CPP data, one of those being the present score measurement. In addition, previous studies exploring ethanol-induced CPP have selected the score measurement and similar scores (between 150 and 200 seconds in a 15 min Test) have been reported for the conditioned groups (Gibula-Bruzda et al., 2015; Walker and Ettenberg 2007).

It is important to note, that, in experiment V, **only the intermediate doses assayed elicit ethanol CPP**, a fact that is in accordance with previous findings. For example, studies using medium to high doses of ethanol such as 1 or 1.5 g/kg **i.p.** and similar experimental design to the one followed in this study, have reported ethanol-place preference (Bahi and Dreyer 2012; Peana et al., 2008). Also, the aforementioned icv study by Walker and Ettenberg points to a similar dose-response curve of ethanol CPP (Walker and Ettenberg 2007).

Very impressively, **ethanol administration** not only can induce CPP but also **can result in an aversion (CPA) to the drug-paired compartment when a higher dose is administered**. That is the effect observed in **experiment V** in the **300 nmol** ethanol treated animals, which show a clear negative preference score (Figure 4.12). There is also previous evidence of ethanol-induced CPA in the literature. Concretely, some studies reported aversion to the ethanol compartment when high systemic ethanol doses (2 g/kg) were administered in both rats (Becker et al., 2006; Zarrindast et al., 2010) and mice (Cunningham and Henderson 2000). According to the metabolites hypothesis on the role of ethanol metabolites in ethanol-derived actions in the MCLS (see section 1.3.2), this **ethanol-induced place aversion could be a consequence of the direct action of the ethanol molecule itself** (i.e., the non-metabolized fraction of the administered dose) on the VTA. As exposed in section 1.3.2, from a kinetic point of view, the percentage of the ethanol administered converted into its

metabolites will depend on the dose. Larger doses, exceeding the capacity of the metabolic system, will render higher levels of the non-metabolized ethanol fraction.

As previously mentioned, this initial set of CPP studies was designed to characterize the ability of intra-VTA ethanol to elicit CPP, with the objective of using this behavioral paradigm to explore the possible effect of inflammatory pain. Nonetheless, given the interesting CPP and CPA obtained results, a deeper analysis of these ethanol context learned associations was also carried out in the present Thesis. Thus, the critical role of local MORs in the VTA and the involvement of the NMDAR subunits in ethanol CPP was explored. In this way, results from **experiment VI** show that the blockade of local VTA MORs during the conditioning phase impaired the acquisition of ethanol place preference. This points to the fact that **MORs in the VTA have a key role in the development of ethanol induced context-learned associations**. Furthermore, these results are in accordance with previous studies that have also showed that acquisition of ethanol-induced CPP (i.p. or icv) can be prevented by inhibiting MORs (Gajbhiye et al., 2017; Gibula-Bruzda et al., 2015; Quintanilla et al., 2014). As explained in section 1.3.2, the activating effects of ethanol could be the result of the **direct action of the ethanol metabolites** (and, concretely, salsolinol) **on the VTA MORs**. According to this metabolites hypothesis, the **products derived from the ethanol metabolism (i.e., salsolinol) would be the responsible for the induction of the place preference** (see section 1.3.2).

Current data together with our and other previous data clearly indicate that understanding the ethanol mechanism of action in the VTA to produce reinforcement or aversion is still unknown and more efforts are needed to shed

light on this important issue. Finally, results from experiment V and experiment VI confirm that the CPP paradigm is appropriate for the study of the effect of pain-induced changes in VTA MORs on DA-dependent behaviors induced by ethanol

Regarding the involvement of the NMDAR subunits, the results from **experiment V** indicate an **increase in NR2A mRNA** dots/cell counts (Figure 4.17), together with a tendency to increased NR2A protein levels expression (Figure 4.13) **in the NAc** of animals that showed ethanol place preference (70 nmol). These changes could be attributed **to the establishment of an association between the ethanol effects and a specific drug-conditioning compartment**, since ethanol-unpaired 70 nmol rats exhibit no changes in NMDAR expression (Figure 4.13). Thus, it could be possible that accumbal NR2A have a key role in the development of the association between ethanol and the environment. In fact, NR2A knockout mice are not able to develop morphine or ethanol CPP (Boyce-Rustay and Holmes 2006; Miyamoto et al., 2004), whereas the blockade of NR2B by administering a selective antagonist does not alter ethanol place preference (Boyce-Rustay and Cunningham 2004). In addition, local antagonism of NMDARs in the NAc before the test session blocks the expression of ethanol induced CPP (Gremel and Cunningham 2009). Unfortunately, the current lack of a specific NR2A antagonist, complicates the study of the concrete role of this subunit in drug-induced context learned associations.

Interestingly, and in line with results **in the hippocampus**, **ethanol place preference does not seem to correlate with changes in the expression of NR1 nor NR2A** subunits (or their corresponding mRNA) in this region (Figure 4.14). This is an intriguing result because previous data have shown

that morphine CPP in mice is related to an increase of NR1 subunit in the hippocampus (Portugal et al., 2014). In addition, the local antagonism of NMDA receptors in the hippocampus has also been able to prevent the acquisition but not the expression of morphine-induced place preference (Zarrindast et al., 2007). However, it is important to note that in these experiments morphine was administered systemically paired to a context, in contrast to the current experiment in which ethanol was locally administered into the VTA paired to the context. Unfortunately, there are no previous studies analyzing the expression of these NMDA subunits in the hippocampus after the development of ethanol CPP. It could be possible then, that these changes in the hippocampus that may occur as a consequence of the association of ethanol to a concrete context could differ from those elicited by other opioidergic drugs or may be related to the drug induced activation of MORs located in other brain areas. Further investigation may help to understand the role of hippocampal NMDAR in the expression of ethanol-induced CPP.

5.2.2.b Effect of inflammatory pain on ethanol-induced CPP

All in all, experiment V and experiment VI succeed to characterize the CPP elicited by the local VTA administration of ethanol, in which local VTA MORs showed to have a critical role. The next question, then, was: does inflammatory pain alter that ethanol-induced CPP? Results from **experiment VII** show that local administration of **52 nmol of ethanol intra-VTA is able to induce preference for the ethanol-paired compartment only in saline rats, but not in CFA rats** (Figure 4.27). And very interestingly, it shows that **a higher ethanol dose of 70 nmol is able to overcome this pain-induced blockade of ethanol place preference** (Figure 4.27), hence resulting in similar preference scores in both saline and CFA rats. Moreover, the Von Frey test data confirms the validity of our inflammatory pain model and that no changes in

the nociception were observed neither in CFA nor saline rats through the CPP experimental process (Figure 4.26). These crucial data allowed us to rule out the possibility of unspecific behavioral effects derived from changes in mechanical nociception. The **shift to the right in the dose-response of intra-VTA ethanol CPP** provides more evidence that **dopamine-dependent behaviors are altered in CFA rats and higher ethanol doses are needed** to elicit comparable effects as in saline rats.

Similar results to these have been previously reported for other drugs of abuse. Concretely, Narita and collaborators showed a blockade of morphine-induced CPP (8 mg/kg, i.p.) in rats suffering inflammatory pain (Narita et al., 2005). This behavior was also correlated with a decrease of morphine-evoked DA release in the NAc in the presence of inflammatory pain. However, in this study none of the two morphine doses administered were able to induce morphine place preference in the inflammatory pain group. Another study, also using a **morphine CPP paradigm**, does, indeed, report a **shift in the dose-response of the drug induced place preference** (Wu et al., 2014). In this case, the development of neuropathic pain in rats only blocked morphine-induced CPP at the lower dose (3.5 mg/kg, s.c.), but not at higher doses (5 mg/kg or 7 mg/kg, s.c.). Very interestingly, the same effect was reported when the selective agonist DAMGO was administered into the VTA in another CPP study (Taylor et al., 2015). Concretely, Taylor and collaborators showed that the place preference elicited by intra-VTA administration of DAMGO was blocked in animals under neuropathic pain only at low doses of DAMGO. Thus, an abnormal function of MORs in the VTA of pain animals appears to be the responsible for the shift in drugs dose-response for CPP. All these observed phenomena are, likewise, consistent with other interesting findings reported in Hipolito et al., 2015. In that study, higher doses of heroin (i.v.) were necessary

to elicit heroin self-administration in CFA-treated rats (Hipolito et al., 2015). This self-administration data are of high importance because they show that **animals, when developing a pain condition, change their pattern of consumption and self-administer opioids only when higher doses are presented**. Therefore, it could be possible that pain condition induces a shift in the dose-response of opioidergic drugs and ethanol, what may be translated into a higher dose drug consumption in pain-suffering patients.

Taken together, results from **experiment VII** show that inflammatory pain alters ethanol reinforcing action on the MCLS which may have consequences in alcohol consumption patterns.

5.3 HOW DOES INFLAMMATORY PAIN IMPACT ON THE ADE IN LONG-TERM EXPERIENCED ANIMALS?

The last concrete objective of the present Thesis was to study whether this previous reported alteration in ethanol-evoked neurochemical responses and dopamine-dependent behaviors induced by inflammatory pain could have an **effect on AUDs related behaviors**. Pain condition frequently elicits negative affective states driving to alterations in reward evaluation, decision making and motivation (Apkarian et al., 2013; Verdejo-Garcia et al., 2009). A similar negative affective state is also present in AUD. Concretely, this state is characteristic of withdrawal phases, and it is related with drug seeking and relapse (Edwards and Koob 2010). Thereupon, the **development of a pain condition during withdrawal could facilitate a negative affective state and thus increase the risk of alcohol seeking and relapse**. Given the recent epidemiological data showing that higher levels of pain correlate with a higher risk of alcohol relapse (Jakubczyk et al., 2016), an alcohol relapse behavioral approach was chosen to investigate this connexion between pain and relapse in a preclinical setting.

The long-term non-operant self-administration paradigm was selected, since it has been widely employed for the study of alcohol relapse-like behaviors in rodents by our and other groups and it has been proved to provide predictive validity (see section 1.3.1.b) (McKinzie et al., 2000; Orrico et al., 2013; Vengeliene et al., 2008).

First, the **paradigm was characterized** to ensure that the correct growth and the normal behavior of the animals was not modified by neither the long exposure to ethanol nor the development of the inflammatory pain condition. With this purpose, **animals weight** was measured and no significant differences

between groups were found before (Figure 4.28) or after (Figure 4.29) the CFA or saline s.c. injection. Moreover, the two selected groups did not differ in **total volume of liquid intake** during the whole exposure prior to CFA or saline injection (Figure 4.30), or after the development of the inflammatory pain condition (Figure 4.31). Finally, the patterns of **total ethanol intake** were also analyzed during the first three cycles of exposure and abstinence to be sure that the possible changes observed after the 4th abstinence period are not derived from differences between the two groups before the injection of CFA or saline. In this way, both groups did not show significant differences in the total ethanol intake before the 4th abstinence period (Figure 4.32). When analyzing the development of an ADE after each ethanol reintroduction (Figure 4.33), results also showed that the two groups significantly increased their total ethanol intake after two of the first three abstinence periods. And, what is more important, both groups developed this ADE during the third cycle, e.g., both groups showed similar relapse-like behavior before the development of the inflammatory pain condition in the CFA selected rats. All this data are of high importance, as they provide evidence that the methodology of the long-term alcohol exposure is not affecting the obtained results.

Thereafter, during the 4th abstinence and post-abstinence periods, the effect of inflammatory pain on relapse was analyzed. As expected, our results indicate that **alcohol-deprived CFA rats developed ADE** after alcohol reintroduction (Figure 4.34 and Figure 4.35). However, **the magnitude of the ADE did not change relative to control rats.**

The fact that there were no differences in alcohol intake between saline and CFA rats may be considered as surprisingly contradictory to the previous clinical data. The existing literature in humans shows that the presence of pain

is correlated with higher risk of alcohol relapse (Jakubczyk et al., 2016) and it also predicts heavy drinking (Witkiewitz et al., 2015b). Thereupon, the expected result in this experiment would have been to observe higher levels of alcohol consumption after the abstinence period in pain suffering animals. Although the present study does not provide a pain-derived effect in ADE, it is **difficult**, however, to **infer that pain does not alter in any way rats relapse-related behaviors**. It is important to consider that relapse is a complex phenomenon and animal models try to reproduce a specific aspect of this behavior. The paradigm chosen for the present Thesis has been used by numerous preclinical studies to test different pharmacological strategies aimed to suppress or reduce ethanol relapse, but it has never been used to show an increase of the risk of relapse (McKinzie et al., 2000; Orrico et al., 2013). The ADE is defined as an increase in total ethanol intake that occurs during the first days after an abstinence period. As this increase normally occurs in the general rat population (control rats in experiment VIII), one possible result for this experiment could have been that the rats treated with CFA had shown an increase in alcohol intake (an ADE) greater than the increase exhibited by the control rats. However, experiment VIII failed to display such increase: **rats in both groups showed similar increases in alcohol intake**. Apparently, these results would suggest that pain did not change the relapse-like behavior in rats as measured in our ADE paradigm.

However, the absence of **changes in the magnitude of the ADE could not fully exclude the existence of changes in alcohol relapse vulnerability**. Another interesting possibility, unfortunately not explored in this Thesis, is that pain does induce an increase in the vulnerability to relapse. In this case, the variable that should have been analyzed should have been the **relapse rate**, that is, the percentage of animals that show ADE after the period of forced

abstinence. Then, if chronic pain has any effect on relapse rate, CFA-treated animals should have shown a **higher rate of relapse** than that observed in control animals. Therefore, further behavioral studies involving a greater number of animals (that allow to investigate alcohol drinking behavior in the face of vulnerability to relapse) are needed and would shed more light in this aspect.

It also is important to consider that **pain-induced changes may differently affect addictive behaviors depending on the different stages of the AUD**. In fact, recent studies using a two-bottle choice paradigm show that pain induction prior to alcohol exposure significantly increases total intake in mice (Butler et al., 2017; Yu et al., 2019). Thus, it may be plausible that pain increases alcohol intake during acquisition without modifying the magnitude of the ADE. On the other hand, there is a recent study in which neuropathic pain induced prior to a two-bottle choice ethanol exposure did not change the total ethanol consumption in mice (Bilbao et al., 2019). Although these three studies were performed under very similar conditions (same mice strain and ethanol exposure paradigm), the ethanol concentration in the two studies that report an increase in ethanol consumption is higher (20% ethanol) (Butler et al., 2017; Yu et al., 2019) that the one used by Bilbao and collaborators (12% ethanol) (Bilbao et al., 2019). This further supports the fact that despite results from experiment VIII do not show differences between saline and CFA rats in the magnitude of the ADE, many methodological factors could be masking a possible pain-induced effect on relapse.

In any case, data from experiment VIII highlight the necessity of finding the appropriate animal model that reflects the existing clinical evidence and allows us to study the alcohol-related behavioral implication of pain-induced

alterations of the MCLS. Moreover, the present research provides relevant data as it **analyses, for the first time, the effect of inflammatory pain on alcohol relapse in animal models** and therefore constitutes an important contribution to the study of pain and AUD.

6 CONCLUSIONS

The most relevant conclusions derived from the data obtained in the present Thesis are:

1. Intra-VTA DAMGO doses that induce cFos expression in the BLA, ACC and BNST are insufficient to activate MORs in CFA-treated animals, suggesting that inflammatory pain desensitizes local MORs in the VTA .

2. Inflammatory pain decreases the ability of the MORs agonist DAMGO in the VTA to increase DA release in the NAc. This effect can be restored with higher doses of the agonist, further supporting pain-induced desensitization of MORs.

3. Inflammatory pain impairs ethanol-evoked DA release in the NAc.

4. Ethanol administration into the VTA is able to induce CPP or CPA depending on the selected dose. The development of the ethanol-context preference necessarily involves the activation of the MORs within the pVTA and modifies the mRNA levels of NR2A in the NAc.

5. Inflammatory pain alters the CPP elicited by ethanol administered into the VTA. Higher doses of ethanol are required to observe a preference for the ethanol associated context in pain suffering animals.

6. Alcohol-deprived rats under inflammatory pain develop ADE after alcohol reintroduction. The magnitude of the ADE, however, does not differ from the control group.

7 RESUM

Segons l'últim "Informe sobre l'estat de l'alcohol i la salut" de l'Organització Mundial de la Salut (OMS), el **trastorn per consum d'alcohol (AUD de les sigles en anglés *Alcohol Use Disorder*)** és el tercer principal risc de malaltia a Europa i és molt resistent a tractaments tant farmacològics com d'altres tipus, especialment en certes situacions clíniques (Litten et al., 2012). L'Oficina Regional de l'OMS a Europa fa èmfasis en la necessitat d'emprar un major esforç en la recerca per aclarir quines són aquestes situacions clíniques especials que augmenten el risc de patir AUD.

D'altra banda, el dolor crònic és un problema de salut molt important, que afecta el 20-30% de la població europea en general. A més, el dolor crònic sovint va acompanyat de trastorns afectius i emocionals, que són molt difícils de tractar (Bair et al., 2013; Bushnell et al., 2013; Fine 2011). Estudis recents en humans i animals van revelar que **el dolor impacta negativament en el processament motivacional i de recompensa, ja que altera el normal funcionament de sistema mesocorticolímbic (SMCL)** (Taylor 2017). Aquest sistema s'encarrega tant de la regulació del comportament de motivació i l'aprenentatge de reforç per recompenses naturals i drogues, com també de la codificació d'estímuls aversius (Everitt and Robbins 2005; Koob and Volkow 2016 ; Salamone 2016). Per tant, és possible que els **efectes del dolor al SMCL no sols afecten a la qualitat de vida dels pacients (per exemple, provocant anhedonia, depressió, estats afectius negatiu), sinó que també tinguen un impacte important en la vulnerabilitat a l'abús de drogues.**

Múltiples estudis clínics i epidemiològics han posat de manifest que la presència de **dolor crònic està estretament relacionada a amb l'AUD (Witkiewitz et al., 2015)**. Tota aquesta evidència apunta al dolor crònic com

un factor que augmenta el risc de patir AUD i en pacients amb antecedents d'abús d'alcohol sembla que promou el consum excessiu d'alcohol i la recaiguda (Jakubczyk et al., 2016 ; Witkiewitz et al., 2015b). No obstant això, i malgrat la rellevància clínica d'aquest fet en la selecció i el disseny de les estratègies per al tractament del dolor i de l'AUD, pocs estudis han examinat els mecanismes i circuits neuronals subjacents a aquesta relació.

Tots els esforços per reduir la prevalença de l'AUD són necessaris, inclosa la recerca per comprendre els factors que promouen el consum excessiu d'alcohol i les recaigudes. Especialment, durant els últims anys, la recerca s'ha centrat en trobar noves estratègies per a la prevenció de la recaiguda, ja que constitueix el principal repte per al tractament dels pacients dependents a alcohol. Per tant, és de gran importància ampliar els coneixements respecte als efectes que el dolor provoca en la recaiguda i, en general, a l'AUD. A més, aquests resultats tindrien una aplicabilitat extrema a la detecció primerenca i la prevenció d'AUDs en pacients amb dolor. Per últim, també podrien ajudar al desenvolupament d'estratègies terapèutiques noves i més segures per tractar el dolor i l'AUD.

Per tot això, l'objectiu general de la present Tesi és **explorar les alteracions induïdes pel dolor inflamatori en la modulació de sistema opioide endogen sobre el SMCL dopaminèrgic** i investigar si aquestes alteracions podrien **afectar a la recaiguda en el consum d'alcohol** en models de comportament animal.

L'addicció a les drogues és una patologia molt complexa, que compren diferents regions de cervell involucrades en la recompensa, la emoció, la presa de decisions i l'hàbit. Concretament, l'efecte reforçant tant dels reforçadors

naturals com de les drogues està controlat pel SMCL. Aquest és un sistema heterogeni, format pel nucli accumbens (NAc), el nucli del llit de l'estria terminal (BNST), l'amígdala central (CeA), l'hipocamp i les regions corticals frontals. Tots ells reben innervació dopaminèrgica de l'àrea tegmental ventral (VTA) del mesencèfal (revisat en Fields et al., 2007). Al SMCL, la senyalització a través de sistema opioide endogen juga un paper clau en la regulació de l'activitat de les neurones de dopamina (DA) del VTA que projecten al NAc. Aquest sistema comprén tres tipus de receptors acoblats a proteïnes G: els receptors mu (MOR), delta (DOR) i kappa (KOR). Concretament, els **MORs són essencials per al processament de recompenses tant de estímuls naturals com de drogues d'abús** (revisat a Le Merrer 2009). L'activació dels MORs ubicats al VTA augmenta l'alliberament de DA a les seues regions de projecció, un mecanisme que tradicionalment s'ha implicat en les propietats de reforç de les drogues, inclòs l'etanol (Charbogne et al., 2014, Darcq i Kieffer 2018; Hipólito et al., 2009; Kieffer i Gavériaux-Ruff 2002; Martí-Prats et al., 2015; Melis et al., 2009; Xiao i Ye 2000; Xie et al., 2012).

Estudis previs suggereixen que el **dolor indueix canvis plàstics al SMCL** (Navratilova i Porreca 2014; Taylor 2017). Concretament, diversos estudis en aquest camp han revelat que en els pacients amb dolor persistent hi ha un augment en la connectivitat corticoestriatal funcional (Baliki et al., 2012) i que tant en humans (Loggia et al., 2014) com en rosegadors (Ozaki et al., 2002; Ren et al., 2015; Taylor et al., 2015), la presència de dolor es correlaciona amb una reducció de la funció dopaminèrgica. També s'ha demostrat que el dolor persistent modifica el comportament motivacional per les drogues (especialment estudiat en opioides) (Massaly et al., 2016; Martin et al., 2007) i per les recompenses naturals (Massaly et al. , 2019; Schwartz et al., 2014). Un dels possibles mecanismes contribuents a aquests esdeveniments

podria ser la disminució en la funció MOR al SMCL. De fet, un gran nombre d'estudis han descrit una disminució a la funció MOR en moltes regions involucrades en el processament del dolor (Aoki et al., 2014; Hurley i Hammond 2001; Jongeling et al., 2009; Shaqura et al., 2004; Zhang et al., 2004). Seria, per tant, lògic, pensar que **la presència de dolor puga comportar també una disminució de la funció dels MORs al SMCL** i, concretament, al VTA.

Per tot això, el primer objectiu concret d'aquesta Tesi (**O1**) va ser **investigar l'efecte del dolor inflamatori en la funció MOR al SMCL**. Amb aquest propòsit, es va seleccionar el model de dolor inflamatori de l'Adjuvant Complet de Freund (CFA) i l'expressió de cFos com una mesura indirecta de l'activació cel·lular. El CFA està compost per Mycobacterium tuberculosi inactivat per calor i suspés en olis no metabolitzables (oli de parafina i monooleat de cacauet). La injecció intraplantar de CFA diluït 50:50 en solució salina a la pota posterior de l'animal indueix inflamació local, tumefacció de les potes i dolor, que persisteixen durant 2-4 setmanes després de la injecció (Chang et al., 2010). La injecció de **CFA** va ser el **model de dolor inflamatori que es va utilitzar en tots els experiments** plantejats en la present Tesi. En relació a l'expressió de cFos, és important recalcar que la detecció d'aquesta proteïna s'ha utilitzat amb freqüència des de finals de 1980 (Bullitt 1990; Dragunow i Faull 1989) per mapificar grups de neurones que mostren canvis en la seua activitat. Per tant, en el primer grup d'experiments, es va triar **l'expressió de cFos per mapificar l'activació d'àrees de projecció del VTA després de l'estimulació local dels MORs**.

Els resultats de l'**experiment I** (Taula 4.1 i Taula 4.2) van mostrar que l'**expressió de cFos a les àrees de projecció del VTA després de**

l'administració intra-VTA de DAMGO (un agonista dels MOR) es redueix o se suprimeix en la majoria de les regions per la presència de dolor inflamatori. En rates control (sense condició dolorosa) (experiment Ia), l'agonisme de MOR localitzat al VTA va augmentar l'expressió de cFos en la majoria de les seues àrees de projecció (NAc, amígdala basolateral (BLA), escorça cingulada anterior (ACC) i BNST), amb l'excepció de l'escorça prelímbica (PL), escorça infralímbica (IL) i pàlid ventral (VP). No obstant això, en les rates sotmeses a la condició de dolor inflamatori (rates CFA, experiment Ib), aquesta resposta va disminuir en la majoria de les regions (BLA, ACC i BNST).

Els resultats obtinguts en l' **experiment Ia** (Taula 4.1) també revelen la complexitat del control opioidèrgic de l'activitat de les projeccions de VTA. Per exemple, en el cas de **NAc** i **BLA** , l'administració de DAMGO 14 ng, però no DAMGO 7 ng, va augmentar significativament el recompte de cèl·lules immunoreactives per cFos (cFos-IR) en comparació amb l'administració de fluid cerebral espinal artificial (aCSF). Per contra, en l'**ACC**, el recompte de cFos-IR va augmentar quan es va administrar DAMGO 7 ng al VTA però no quan es va injectar DAMGO 14 ng. Per contra, ni PL ni IL es van activar significativament després de l'administració de cap de les 2 dosis. De manera similar a l'ACC, la dosi baixa de DAMGO va augmentar els recomptes de cFos-IR al **BNST**. Finalment, en el cas del VP, l'activació dels MORs al VTA no va augmentar l'expressió de cFos.

Curiosament, els resultats de l'**experiment Ib** (Taula 4.2) mostren que la presència de **dolor inflamatori altera l'augment anteriorment descrit** de l'expressió de cFos induïda per DAMGO intra-VTA en totes les àrees analitzades, excepte en el NAc. Estudis previs ja han demostrat que la

dessensibilització induïda pel dolor en els MORs del VTA té un efecte sobre l'activitat del NAc (Ozaki et al., 2002). Sorprenentment, en el present estudi, l'administració de 14 ng de DAMGO tant en rates control com en rates CFA va produir un augment en el recompte de cFos-IR en el NAc. Donats les troballes esmentades anteriorment, un resultat més esperat hauria estat que la dosi que augmenta significativament el recompte de cFos-IR en el NAc dels animals de control (14 ng de DAMGO) no induïsca aquest augment en les rates CFA. Això hauria recolzat el fet que la dessensibilització dels MORs locals en el VTA té conseqüències en l'activació, induïda per opioides, de les neurones de DA que projecten al NAc.

Diverses raons poden ser les que ens impedeixen observar un canvi en la corba dosi-resposta en el NAc en aquest experiment. En primer lloc, el fet de no haver detectat un efecte per a la dosi més baixa de DAMGO en l'experiment Ia (rates control) és prou rellevant. En aquest grup, l'administració de DAMGO 7 ng va provocar un augment de cFos-IR en el NAc molt similar al provocat per la dosi de DAMGO 14 ng, la qual cosa no passa en el grup amb dolor. No obstant això, aquests augments no van ser estadísticament significatius en cap de les dues condicions experimentals (rates control o CFA). En segon lloc, és possible que 14 ng ja siga una dosi prou alta per activar les denses projeccions del VTA al NAc en animals amb dolor, tot i havent una menor funció dels MORs en el VTA. En aquest cas, és possible que una dosi intermèdia de DAMGO siga útil per detectar un augment de l'expressió de cFos sols en animals control. I tercer, és important tindre en compte que l'expressió de cFos és el resultat de diversos esdeveniments que tenen lloc durant un llarg període de temps, i no només una conseqüència directa de l'augment en l'alliberament de DA. Per tant, és també possible que, en rates CFA, 14 ng de DAMGO administrat intra-VTA resulte en una activació al

NAc similar a la de les rates control, encara que els resultats de comportament podrien diferir radicalment.

Per contra, en les altres regions estudiades, el dolor inflamatori si que va alterar el patró de recompte de cFos-IR observat en l'experiment Ia després de l'administració de 7 o 14 ng de DAMGO. De fet, en presència de dolor inflamatori, **l'administració de DAMGO intra-VTA no va induir una activació de BLA, ACC i BNST**. Per tant, les dosis de DAMGO que augmenten l'expressió de cFos a les regions de projecció en l'experiment Ia són insuficients per induir l'expressió de cFos en rates CFA .

A la llum d'aquests resultats, l'**experiment II** i l'**experiment III** es van dissenyar amb l'objectiu d'**explorar més a fons l'efecte del dolor inflamatori en la funció moduladora dels MORs locals** (tant en el NAc com en el VTA), mesurant l'alliberament de DA al NAc. Per a això, es van realitzar estudis de **microdiàlisi *in vivo***, ja que aquesta tècnica permetia mesurar l'efecte de diferents tractaments sobre els nivells de DA en el NAc de les rates. L'alliberament de DA a la via VTA-NAc es clau en les propietats de reforç de drogues i reforçadors naturals i aquest alliberament de DA està estrictament regulat pels MORs en aquesta via (Devine et al., 1993; Johnson i North 1992; Le Merrer et al., 2009; Olszewski et al., 2011; Wassum et al., 2009; Wise et al., 1995). Com s'ha comentat amb anteriorment, hi ha evidències que suggereixen que el dolor altera la funció dels MORs al SMCL, de manera que els increments de DA al NAc induïts per morfina no es produeixen en animals amb dolor neuropàtic (Ozaki et al., 2002). Per tant, tot i no observar canvis induïts pel dolor a la activació dels MOR d'aquest circuit per la mesura d'expressió de cFos (experiment I, resultats en NAc), encara seria possible trobar diferències en l'alliberament de DA provocada pels agonistes del MOR.

D'aquesta manera, els resultats de l'experiment III (Figura 4.7) van revelar que, **en animals amb dolor, el DAMGO (7 ng en el VTA) té reduïda la capacitat d'augmentar l'alliberament de DA al NAc**, la qual cosa suggereix una disminució de la funció dels MORs. Aquestes troballes concorden amb investigacions prèvies que han mostrat que el dolor indueix un deteriorament d'activitat dopaminèrgica mesolímbica. Concretament, estudis d'imatge en humans amb dolor crònic han trobat que la resposta del SMCL dopaminèrgic a estímuls de reforç és menor en comparació amb subjectes sans (Loggia et al., 2014; Taylor et al., 2016). També estudis en animals corroboren aquest fet, ja que mostren disminucions en l'alliberament de DA provocat per opioides en rosegadors que pateixen dolor (Ozaki et al. 2002; Taylor et al., 2015).

Interessantment, l'administració intra-VTA d'una dosi prop a 10 vegades més gran de DAMGO (67 ng) va augmentar significativament l'alliberament de DA tant en rates control com en rates CFA (Figura 4.7). Aquesta troballa apunta a un possible canvi en l'efecte del DAMGO dependent de la dosi, la qual cosa dóna encara més suport al fet que les rates CFA tenen disminuïda la funció dels MORs locals del VTA. De fet, aquest efecte ja s'havia mostrat prèviament en les regions cerebrals involucrades en la nocicepció (Aoki et al., 2014; Hurley i Hammond 2001; Jongeling et al., 2009). A més, Ozaki i col·laboradors (Ozaki et al., 2002), al combinar les tècniques de microdiàlisi i GTPγS, van concloure que, en presència de dolor neuropàtic, les rates van mostrar una pèrdua de l'alliberament de DA induïda per morfina en el NAc, a causa de la reducció en la funció dels MORs al VTA. Per tant, com ocorre a l'experiment III, **en el VTA es necessiten dosis més altes d'agonista per superar la dessensibilització induïda pel dolor dels MORs i provocar augments similars a l'alliberament de DA als quals es produeixen en els animals control** .

D'altra banda, en l'**experiment II** també es va estudiar l'efecte de l'**administració intra-NAc de DAMGO 10 μM**. Els resultats, en aquest cas, **no van mostrar diferències significatives en els nivells de DA entre els grups control i CFA** (Figura 4.5). Aquests resultats suggeririen que l'alteració de la modulació dels MORs sobre la transmissió de DA que indueix el dolor es va localitzar principalment en el VTA, i no en el NAc. Per contra, també és possible que la dosi seleccionada en aquest cas no siga l'adequada per observar diferències entre grups. De fet, en les rates control aquesta dosi de DAMGO produeix augments significatius en els nivells de DA respecte els seus nivells basals. No obstant això, en els animals sense dolor, cap dels valors de nivell de DA posteriors a l'administració de DAMGO 10 μM va augmentar significativament en comparació al nivell basal. És curiós com, d'altra banda, l'anàlisi estadístic no va mostrar diferències entre els dos grups. Per tant, és probable que, usant una dosi menor de DAMGO, si que es produïsca una diferència significativa entre els grups, de manera semblant a l'experiment III.

En conjunt, aquests resultats demostren que el **dolor inflamatori produeix una dessensibilització dels MORs del VTA i, per tant, que la regulació de les neurones de DA del VTA que innerven diferents àrees de projecció està alterada en els animals amb dolor**.

Diverses investigacions realitzades en les darreres dècades evidencien que les propietats de reforç d'etanol estan, al menys en part, regulades pels MORs del SMCL (Guan i Ye 2010; Sánchez-Catalán et al., 2009 Xiao et al., 2007; Xiao i Ye 2008). Aquest fet, juntament amb les troballes prèvies que demostren una pèrdua de la funció dels MOR del VTA en rates CFA, va donar lloc al segon objectiu concret d'aquesta Tesi (**O2**): **estudiar les conseqüències**

neuroquímiques i conductuals provocades pel dolor inflamatori en les propietats de reforç de l'etanol .

Per a això es va plantejar, en primer lloc, un experiment de **microdiàlisi** (**experiment IV**) per estudiar l'**efecte del dolor inflamatori sobre l'alliberament de DA al NAc induït per l'administració d'etanol sistèmic** (1.5 g/kg, subcutani (s.c.)). Com a resultat, aquesta administració sistèmica d'**etanol**, que si que va produir **un augment significatiu en els nivells de DA en el NAc de les rates control, no va aconseguir provocar l'augment de l'alliberament de DA en el cas de les rates CFA** (Figura 4.9). És important destacar que els estímuls estressants aguts també augmenten els nivells de DA en el NAc (Abercrombie et al., 1989). Per aquesta raó, en aquest experiment, les rates van rebre una injecció prèvia de solució salina (s.c.) amb l'objectiu de discriminar aquest efecte induït per l'estrés. Tot i que els nivells de DA després d'aquesta injecció prèvia van ser més alts que els valors inicials tant en rates control com en rates CFA, aquest augment no va ser estadísticament significatiu per a cap dels grups (Figura 4.9). A més, **l'efecte global provocat per l'etanol en rates control va ser significativament més gran que l'efecte total induït per solució salina** (Figura 4.10). Per contra, **l'efecte total induït per solució salina i per etanol no van ser estadísticament diferents en les rates CFA**, la qual cosa confirma que els resultats d'aquest experiment deriven de les propietats farmacològiques de la droga.

Els resultats de l'experiment IV, donen encara més suport al fet que el dolor inflamatori altera la resposta neuroquímica del SMCL provocada per les drogues i, concretament, per l'etanol. De manera similar, investigacions prèvies han mostrat una disminució en l'alliberament de DA evocada per opioides en el NAc de rates en condicions de dolor (Hipólito et al., 2015; Ozaki et al., 2002).

Concretament, Hipólito i col·laboradors, mitjançant l'ús del mateix model de dolor inflamatori que el present treball, van demostrar que l'augment de l'alliberament de DA al NAc provocat per heroïna intravenosa (i.v.) disminueix a les rates CFA (Hipolito et al., 2015). En conjunt, aquests resultats de microdiàlisi mostren clarament que el **dolor inflamatori bloqueja l'alliberament de DA provocada per etanol en el NAc**, la qual cosa pot tenir un efecte sobre les propietats reforçants d'aquesta droga.

Està tradicionalment acceptat que els comportaments relacionats amb la recompensa de drogues (com la **preferència de lloc condicionada (CPP)**) estan regulats per la transmissió de DA dins del NAc (Spyraki et al., 1983; Young et al. , 2014). En aquest paradigma, els animals reben una dosi de la droga i immediatament se'ls confina en un context específic; aquest procés es repeteix durant diverses sessions consecutives. Posteriorment, es mesura la preferència de l'animal pel context associat amb la droga en comparació amb un context neutral. Considerant que l'**alteració de la funció neuroquímica del SMCL podria traduir-se en canvis anormals d'aquest comportament**, el CPP va ser el paradigma seleccionat per continuar amb l'**O2** . A més, aquest paradigma s'ha utilitzat prèviament per estudiar les alteracions induïdes pel dolor en l'activació mesolímbica dels opioides (Narita et al., 2005; Ozaki et al., 2002). No obstant això, quan s'ha avaluat la preferència associada a l'administració d'etanol, els resultats han estat paradoxals. De fet, estudis previs en rates han reportat CPP, aversió de lloc condicionada (CPA) o cap canvi en la preferència quan l'etanol es va administrar per rutes sistèmiques (Bahi i Dreyer 2012; Becker et al., 2006; Peana et al., 2008; Zarrindast et al., 2010). A més, Walker i Ettenberg van mostrar que l'administració d'etanol intracerebroventricular (icv.) va ser capaç d'induir CPP a la dosi de 180 nmol, mentre que 120 o 240 nmol no van induir canvis en la preferència (Walker i

Ettenberg 2007). S'accepta clàssicament que l'acció reforçant de les drogues que provoca la preferència per l'entorn associat requereix l'augment de l'activitat neuronal dopaminèrgica del VTA (Bozarth i Wise 1981; Spyraki et al., 1983; Wise 2004). No obstant això, **cap d'aquests o altres estudis previs que utilitzen el paradigma CPP ha administrat etanol directament en regions concretes del SMCL** .

D'altra banda, el **desenvolupament i l'expressió d'associacions apreses entre droga i context s'ha relacionat clàssicament amb la transmissió glutamatèrgica i les formes de plasticitat neural dependents de glutamat induïdes per drogues** (Hearing et al., 2018). Els receptors de glutamat comprenen dues grans famílies, els canals iònics dependents de lligand (receptors ionotròpics) i els receptors dependents de proteïnes G (receptors metabotròpics). Els receptors ionotròpics es divideixen, al seu torn, en tres classes: receptors d'àcid amino-3-hidroxi-5-metil-4-isoxazol propiònic (AMPAR), receptor de kainat i receptors de N -metil-D-aspartat (NMDAR). Els NMDARs, els quals juguen un paper important en la plasticitat sinàptica, són heterotetràmers compostos per dues classes de subunitats: dues subunitats NR1 i dues subunitats NR2. (Lüscher i Malenka 2012). Concretament, els NMDAR participen en el desenvolupament de comportaments induïts pel context tant en el cas de recompenses naturals (Brigman et al., 2013; Dang et al., 2006; Parker et al., 2011; Yin et al., 2008) com en el de les drogues (Beutler et al., 2011b; Daglish et al., 2001; Ersche et al., 2006; Hearing et al., 2017; Heusner i Palmiter 2005; Prosser et al., 2006). No obstant això, **molt pocs estudis han avaluat què paper juga la composició en subunitats del NMDAR en el CPP induït per etanol** .

Derivat de la falta d'estudis de CPP que analitzen l'efecte dosi-resposta de l'administració local d'etanol al VTA, va ser necessari dissenyar un **conjunt inicial d'experiments** abans de la introducció de la variable de dolor inflamatori. Encara que es podria haver seleccionat una altra ruta, es va triar l'administració localitzada al VTA, ja que els resultats abans esmentats d'aquesta Tesi i altres treballs relacionats ja havien demostrat una dessensibilització dels MOR locals de l'VTA induïda per dolor. Per tot això, es van planificar uns estudis inicials amb l'objectiu de (i) demostrar si l'administració local d'etanol al VTA pot induir CPP en rates (ii) trobar la dosi adequada d'etanol intra-VTA que provoca CPP, (iii) explorar la implicació dels MORs al CPP induït per etanol intra-VTA i (iv) analitzar els canvis en l'expressió de subunitats NMDAR en animals condicionats amb etanol.

D'aquesta manera, es va realitzar un primer experiment (**experiment V**) per a l'estudi de la corba dosi-resposta de l'etanol (intra-VTA) per al desenvolupament de CPP. Els resultats mostren, per primera vegada, que l'administració **intra-VTA de 70 i 150 nmol de dosi d'etanol en un context específic dóna com a resultat la preferència per aquest entorn associat a la droga** (Figura 4.12) .

És important tindre en compte que, en l'experiment V, sols les dosis intermèdies d'etanol provoquen CPP, la qual cosa està d'acord amb les troballes anteriors que han emprat aquest paradigma però administrant l'etanol a nivell sistèmic. De fet, els estudis que fan servir dosis mitjanes d'etanol, com 1.5 g/kg intraperitoneal (i.p.) i un disseny experimental semblant a què es va seguir en aquest estudi, mostren una preferència de lloc induïda per etanol (Bahi i Dreyer 2012; Peana et al., 2008). A més, l'estudi icv. esmentat anteriorment per

Walker i Ettenberg apunta a una corba de dosi-resposta similar de CPP d'etanol (Walker i Ettenberg 2007).

Sorprenentment, l'administració d'**etanol** no sols indueix CPP sinó que **també pot donar lloc a una aversió (CPA) al compartiment associat amb la droga quan s'administra una dosi més alta**. Aquest és l'efecte observat en l'**experiment V** en els animals tractats amb **300 nmols** d'etanol (Figura 4.12), que mostren una clara puntuació de CPP negativa. També la literatura presenta evidències prèvies de CPA induït per etanol. Concretament, en alguns estudis, les rates mostren una aversió al compartiment d'etanol quan se les va administrar altes dosis d'etanol sistèmic (2 g/kg) (Becker et al., 2006; Zarrindast et al., 2010).

Totes aquestes dades recolzen una hipòtesi prèviament explorada en el nostre laboratori, segons la qual l'**equilibri entre la presència de la molècula d'etanol i els seus productes metabòlics al SMCL determinaria les propietats activants i reforçants de l'etanol**. Així doncs, sembla que l'activació de les neurones de DA del VTA que s'observa després de l'administració d'etanol és, al seu torn, provocada pels seus productes metabòlics en el cervell (concretament per un seus metabòlits, el salsolinol). D'aquesta manera, els **productes derivats del metabolisme de l'etanol (com ara el salsolinol) serien els responsables de la inducció de la preferència de lloc**. De manera oposada, el CPA induït per etanol **podria ser una conseqüència de l'acció directa de la pròpia molècula d'etanol** (és a dir, la fracció no metabolitzada de la dosi administrada) **al VTA** . Des d'un punt de vista cinètic, el percentatge de l'etanol administrat convertit en els seus metabòlits dependrà de la dosi. Dosis més grans, que excedeixen la capacitat del sistema metabòlic, produiran nivells més alts de la fracció d'etanol no metabolitzada.

Com s'ha comentat anteriorment, aquest conjunt inicial d'estudis de CPP va ser dissenyat per a caracteritzar la capacitat de l'etanol intra-VTA de provocar CPP, amb l'objectiu d'utilitzar aquest paradigma conductual per explorar el possible efecte del dolor inflamatori. No obstant això, considerant l'elevat interés dels resultats obtinguts de CPP i CPA, en la present Tesi també es va realitzar una anàlisi més profunda d'aquestes associacions amb el context induïdes per etanol. Concretament, es va explorar el paper dels MORs locals en el VTA i la participació de les subunitats del NMDAR en el CPP induït per etanol.

D'aquesta manera, els resultats de l'**experiment VI** mostren que **el bloqueig dels MOR locals del VTA** (mitjançant l'administració de **β**-FNA, un antagonista MOR) **durant la fase de condicionament va impedir l'adquisició del CPP induït per etanol** (Figura 4.23). Això apunta el fet que els MORs al VTA tenen un paper clau en el desenvolupament d'associacions amb el context induïdes per etanol. A més, aquests resultats concorden amb estudis previs que també han demostrat que l'adquisició de CPP induïda per etanol (i.p. o icv.) pot prevenir-se inhibint els MORs (Gajbhiye et al., 2017; Gibula-Bruzda et al., 2015; Quintanilla et al., 2014). A més, aquests resultats confirmen que el paradigma de CPP és apropiat per a l'estudi de l'efecte dels canvis induïts pel dolor en els MOR del VTA en els comportaments dependents de DA induïts per etanol.

Com s'ha comentat anteriorment, els NMDAR i les seues diferents subunitats semblen tindre un paper important en els comportaments d'aprenentatge d'associacions entre els efectes de les drogues i l'entorn. Per això, el **teixit cerebral** dels animals dels **experiments V i VI es va analitzar per**

western blot amb l'objectiu de mesurar els nivells d'expressió de les subunitats **NR1** i **NR2A** en àrees cerebrals d'interés (Figura 4.13, Figura 4.14 i Figura 4.24). A l'experiment V, els animals que van adquirir una preferència pel lloc associat a etanol (70 nmol) van mostrar una tendència d'augment en l'expressió de la subunitat NR2A en el NAc, que, però, no era estadísticament significativa al comparar-la amb els animals control (aCSF). A la llum d'aquest resultat es va repetir una cohort d'animals que es van condicionar de nou amb etanol 70 nmol i aCSF (grup control) (Figura 4.16) amb l'objectiu d'utilitzar la nova tècnica de **hibridació *in situ* per RNAscope** i quantificar els nivells d'**ARNm** d'aquestes subunitats. Els resultats d'aquesta anàlisi van mostrar que en les rates condicionades amb etanol 70 nmol es va produir un augment del nºpunts/cèl·lula de l'ARNm de NR2A en el NAc (Figura 4.17). És notable que aquests canvis són atribuïbles a l'establiment d'una associació entre els efectes de l'etanol i un compartiment específic, ja que el western blot no va mostrar cap canvi en l'expressió NMDA en animals que van rebre etanol 70 nmol associat en cada sessió a un compartiment diferent (grup desemparellat). Per tant, podria ser que el possible increment en els nivells de **NR2A en el NAc** tinga un **paper clau en el desenvolupament de l'associació entre l'etanol i l'entorn**. De fet, els ratolins knockout NR2A no desenvolupen CPP per morfina o etanol (Boyce-Rustay i Holmes 2006; Miyamoto et al., 2004), mentre que el bloqueig de NR2B mitjançant l'administració d'un antagonista selectiu no altera la preferència de lloc de l'etanol (Boyce-Rustay i Cunningham 2004). A més, l'antagonisme local dels NMDAR en el NAc abans de la sessió de test bloqueja l'expressió de CPP induït per etanol (Gremel i Cunningham 2009). Desafortunadament, la manca actual d'un antagonista específic per a NR2A complica l'estudi del paper concret d'aquesta subunitat en l'aprenentatge d'associacions entre droga i context .

Curiosament i en línia amb els resultats obtinguts en l'**hipocamp**, la preferència de lloc d'etanol **no sembla induir canvis en l'expressió de les subunitats NR1 ni NR2A** (o el seu **ARNm** corresponent) en aquesta regió (Figura 4.14 i Figura 4.18). Aquest resultat és prou interessant, ja que dades prèvies han demostrat que el CPP de morfina en ratolins està relacionat o amb un augment de la subunitat NR1 en l'hipocamp (Portugal et al., 2014). A més, l'antagonisme local dels receptors NMDA en l'hipocamp també ha estat capaç de prevenir l'adquisició, però no l'expressió de la preferència de lloc induïda per morfina (Zarrindast et al., 2007). No obstant això, és important tindre en compte que en aquests experiments l'aparellament de la morfina amb un context es va realitzar mitjançant administració sistèmica, la qual cosa contrasta amb el present experiment en què l'etanol es va administrar de manera localitzada en el VTA. Malauradament, no hi ha estudis previs que analitzen l'expressió d'aquestes subunitats NMDA en l'hipocamp després del desenvolupament de CPP per etanol. És possible, llavors, que els canvis que es puguen esdevenir en l'hipocamp com a conseqüència de l'associació de l'etanol a un context concret diferisquen dels provocats per altres drogues opioidèrgiques o que estiguen relacionats amb l'activació induïda per drogues sobre els MORs ubicats a altres àrees del cervell. El desenvolupament d'estudis addicionals ajudaria a comprendre el paper dels NMDAR de l'hipocamp en l'expressió de CPP induïda per etanol.

En conjunt, l'experiment V i l'experiment VI van aconseguir caracteritzar el CPP provocat per l'administració local d'etanol al VTA i demostrar el paper essencial dels MORs en aquest comportament dependent de DA. La següent pregunta, llavors, va ser: **el dolor inflamatori altera aquest CPP induït per etanol?** Per a això es va plantejar l'experiment VII que utilitzava el mateix paradigma que els experiments anteriors, administrant

etanol al VTA però incloent, a més, la variable dolor inflamatori. Els resultats de l'**experiment VII** mostren que l'administració local de **52 nmol d'etanol intra-VTA pot induir preferència pel compartiment associat a etanol sols en rates control**, però **no en rates CFA** (Figura 4.27). I el que és més interessant, **una dosi més alta d'etanol de 70 nmol és capaç de revertir aquest bloqueig induït pel dolor de la preferència de lloc d'etanol**, resultant en puntuacions de CPP similars en rates control i CFA. A més, les dades de la prova de Von Frey (prova per mesurar els nivells de dolor mecànic en rata) confirmen que no es van observar canvis en la nocicepció en rates CFA o control al llarg de tot el procés experimental de CPP (Figura 4.26), descartant la possibilitat d'efectes de comportament inespecífics derivats de canvis en la nocicepció mecànica. El canvi en la corba dosi-resposta de l'etanol intra-VTA per produir CPP proporciona més evidència de que els comportaments dependents de la DA estan alterats en rates CFA i es necessiten dosis més altes d'etanol per provocar efectes comparables als que es produeixen en rates control.

Prèviament s'han reportat resultats similars a aquests per a altres drogues d'abús. Concretament, Narita i col·laboradors van mostrar un bloqueig del CPP induït per morfina (8 mg/kg, i.p.) en rates amb dolor inflamatori (Narita et al., 2005). Aquest comportament també es va correlacionar amb una disminució de l'alliberament de DA al NAc evocat per morfina en presència de dolor inflamatori. No obstant això, en aquest estudi cap de les dues dosis de morfina administrades van ser capaces d'induir preferència pel lloc associat a morfina en el grup de dolor inflamatori. Un altre estudi, que també utilitza un paradigma de CPP de morfina, mostra, de fet, un canvi en la resposta de preferència induïda per drogues segons la dosi (Wu et al., 2014). En aquest cas, la presència de dolor neuropàtic va bloquejar el CPP induït per morfina únicament per a la dosi més baixa (3,5 mg/kg, s.c.), però no en el cas de dosis

més altes (5 mg/kg o 7 mg/kg, s.c.). Cal ressaltar també que el mateix efecte en el CPP es va observar per a l'administració de l'agonista selectiu dels MORs, DAMGO, al VTA (Taylor et al., 2015). Concretament, Taylor i col·laboradors van mostrar que la preferència de lloc provocada per l'administració intra-VTA de DAMGO no es va produir en animals amb dolor neuropàtic sols en les dosis baixes de DAMGO. Per tant, la funció anormal dels MOR al VTA en animals amb dolor sembla ser la responsable del canvi en la resposta de CPP per a diferents dosis de la droga. Tots aquests fenòmens són, al seu torn, consistents amb altres troballes interessants reportades per Hipólito i col·laboradors. En aquest estudi, es van necessitar dosis més altes d'heroïna (i.v.) per provocar l'autoadministració d'heroïna en rates tractades amb CFA (Hipólito et al., 2015). Aquestes dades d'autoadministració són de gran importància perquè mostren que els animals, quan desenvolupen una condició de dolor, canvien el seu patró de consum i s'autoadministren opioides només quan es presenten dosis més altes. Per tant, **podria ser que la condició de dolor induïsca un canvi en la dosi-resposta de les drogues opioidèrgiques i l'etanol, la qual cosa pot traduir-se en un consum de dosis més altes de drogues en pacients amb dolor** .

En conjunt, els resultats de l'experiment VII mostren que el dolor inflamatori altera l'efecte reforçant de l'etanol sobre el SMCL, la qual cosa pot tindre conseqüències en els patrons de consum d'alcohol. Tant aquests resultats com els obtinguts en els estudis neuroquímics apunten que el dolor podria tindre un efecte en els comportaments d'AUD. Per tant, el tercer objectiu (**O3**) d'aquesta tesi va ser el d'**explorar l'efecte del dolor inflamatori en la recaiguda en el consum d'alcohol, concretament a l'Efecte de Privació d'Alcohol (ADE) en animals exposats a llargs períodes d'alcoholització**.

La condició de **dolor** freqüentment provoca **estats afectius negatius** que condueixen a alteracions en l'avaluació de recompenses, la presa de decisions i la motivació (Apkarian et al., 2013; Verdejo-Garcia et al., 2009). **També l'AUD** es caracteritza per la presència d'estats afectius negatius similars. Concretament, aquest estat és **característic de les fases d'abstinència**, i està relacionat amb la cerca i la recaiguda en el consum de drogues (Edwards i Koob 2010). Així, el desenvolupament d'una condició de dolor durant l'abstinència podria facilitar un estat afectiu negatiu i, per tant, augmentar el risc de cerca d'alcohol i recaiguda. La prevenció de la recaiguda durant llargs períodes d'abstinència és avui dia el principal problema clínic al qual s'enfronten les estratègies terapèutiques per al tractament de pacients dependents de l'alcohol després de la desintoxicació (Johnson 2008). Per això, i considerant les recents dades epidemiològiques que mostren que els nivells més alts de dolor es correlacionen amb un major risc de recaiguda en l'alcohol (Jakubczyk et al., 2016), es va triar un model animal de recaiguda d'alcohol per investigar aquesta connexió entre el dolor i la recaiguda en un entorn preclínic. Concretament, es va seleccionar l'estudi de l'ADE a un model d'autoadministració no operant a llarg termini, ja que ha estat àmpliament utilitzat per a l'estudi de comportaments similars de recaiguda a alcohol en rosegadors, tant al nostre laboratori com en altres grups, i s'ha demostrat que proporciona validesa predictiva (McKinzie et al., 2000; Orrico et al., 2013; Vengeliene et al., 2014). L'ADE és un comportament característic de la recaiguda que es defineix com un augment robust però transitori en la ingesta de la droga després d'un període d'abstinència forçada. A l'**experiment VIII**, les rates es van sotmetre a un **protocol a llarg termini que va incloure períodes de consum i períodes d'abstinència** distribuïts a l'atzar al llarg del protocol experimental. Aquesta alternança permet reproduir fortament el fenòmen de recaiguda que ocorre

després de cada reintroducció de l'etanol i avaluar-ho a través del mesurament de l'ADE.

En primer lloc, es va realitzar la **caracterització del paradigma** per garantir que ni la llarga exposició a etanol ni la inducció del dolor inflamatori van alterar el creixement o el comportament normal dels animals. Com s'esperava, el pes dels animals, el qual es va registrar al llarg de tot l'experiment, va incrementar de manera gradual i no es van observar diferències entre els dos grups experimentals abans o després de la injecció de CFA o solució salina per les rates control (Figura 4.28 i Figura 4.29). A més, tampoc abans o després de la inducció de dolor es van observar canvis en la ingesta total de líquid entre els dos grups seleccionats (Figura 4.30 i Figura 4.31). Finalment, els patrons d'ingesta total d'etanol també es van analitzar durant els primers tres cicles de exposició i abstinència, per assegurar que els possibles canvis observats després del quart període d'abstinència no foren derivats les diferències entre els dos grups abans de la inducció del dolor. D'aquesta manera, els dos grups no van mostrar diferències significatives en la ingesta total d'etanol abans del quart període d'abstinència (Figura 4.32). Respecte a l'anàlisi del desenvolupament del fenòmen ADE després de cada reintroducció d'etanol, els resultats també van mostrar que els dos grups van augmentar significativament el seu consum total d'etanol després de dos dels tres primers períodes d'abstinència (Figura 4.33). I, el que és més important, els dos grups van desenvolupar aquest ADE durant el tercer cicle.

Finalment, **durant l'últim període d'abstinència es va induir la condició dolorosa** en la meitat de les rates per estudiar l'efecte del dolor inflamatori en el desenvolupament de l'ADE. Com s'esperava, els nostres resultats indiquen que les **rates CFA privades d'alcohol van desenvolupar**

ADE després de la reintroducció d'alcohol (Figura 4.34 i Figura 4.35). No obstant això, la magnitud de l'ADE no va canviar en relació amb les rates control.

El fet que no hi va haver diferències en la ingesta d'alcohol entre les rates control i CFA pot considerar-se sorprenentment contradictori amb les dades clíniques anteriors. La literatura existent en humans mostra que la presència de dolor está correlacionada amb un major risc de recaiguda en el consum d'alcohol (Jakubczyk et al., 2016) i que també prediu el consum excessiu d'alcohol (Witkiewitz et al. 2015b). Per això, el resultat esperat en aquest experiment hauria estat que els animals amb dolor presentaren nivells més alts de consum d'alcohol després del període d'abstinència. **Tot i que el present estudi no proporciona un efecte derivat del dolor en l'ADE, és difícil, però, inferir que el dolor no altera de cap manera els comportaments relacionats amb la recaiguda en rates.** És important recordar que la recaiguda és un fenòmen complex i els models animals intenten reproduir un aspecte específic d'aquest comportament. El paradigma triat per a la present Tesi ha estat utilitzat per nombrosos estudis preclínics per provar diferents estratègies farmacològiques destinades a suprimir o reduir la recaiguda en el consum d'alcohol, però mai s'ha utilitzat per mostrar un augment del risc de recaiguda (McKinzie et al., 2000; Orrico et al., 2013). L'ADE es defineix com un augment en la ingesta total d'etanol que passa durant els primers dies després d'un període d'abstinència. Com aquest augment passa normalment en la població general de rates, en aquest estudi un comportament recaiguda més intens deuria haver-se manifestat com nivells més alts de consum en rates CFA, en comparació amb el grup control. No obstant això, l'experiment VIII no va mostrar tals diferències. També és cert que en aquest estudi tots els animals, tant en el grup control com en l'experimental, presenten un comportament

característic de recaiguda, és a dir, tots dos grups mostren un ADE. D'aquesta manera, els **canvis en la magnitud d'aquest ADE no serien del tot representatius dels canvis en la vulnerabilitat a la recaiguda** en el consum d'alcohol. És possible que el dolor no induïsca una ingesta encara més elevada d'etanol, però si que augmente la vulnerabilitat o el risc de recaiguda. En aquest cas, tot i no produir diferències en el consum, seria plausible esperar majors taxes de recaiguda en animals que pateixen dolor. Per tant, es necessari dur a terme estudis de comportament que permeten investigar la vulnerabilitat a la recaiguda en el consum d'alcohol i que puguen llançar més llum en aquest aspecte.

També és important tindre en compte que els canvis induïts pel dolor poden afectar de manera diferent els comportaments addictius depenent de les diferents etapes de l'AUD. De fet, estudis recents que utilitzen un paradigma de dues botelles mostren que la inducció del dolor abans de l'exposició a l'alcohol augmenta significativament la ingesta total en ratolins (Butler et al., 2017; Yu et al., 2019). Per tant, pot ser **plausible que el dolor augmente la ingesta d'alcohol durant l'adquisició sense modificar la magnitud de l'ADE**. D'altra banda, hi ha un estudi recent en què el dolor neuropàtic induït abans d'una exposició etanol en un paradigma de de dues botelles no va canviar el consum total d'etanol en ratolins (Bilbao et al., 2019). Encara que aquests tres estudis es van realitzar en condicions molt similars (mateixa soca de ratolins i mateix paradigma de exposició a l'etanol), la concentració d'etanol en els dos estudis que mostren un augment en el consum d'etanol és més gran (20 % d'etanol) (Butler et al., 2017; Yu et al., 2019) que la utilitzada per Bilbao i col·laboradors (12% d'etanol) (Bilbao et al., 2019). Això dóna suport al fet que, tot i que els resultats de l'experiment VIII no mostren diferències entre les rates control i CFA, molts factors metodològics podrien estar emmascarant un

possible efecte induït pel dolor a la recaiguda. A més, cap dels estudis esmentats analitza el dolor com un factor de risc de recaiguda.

En qualsevol cas, les dades de l'experiment VIII destaquen la necessitat de trobar el model animal apropiat que reflectisca l'evidència clínica existent i ens permeta estudiar la implicació de les alteracions induïdes per dolor del SMCL en les conductes relacionades amb l'AUD. A més, la present Tesi proporciona dades rellevants, ja que **analitza, per primera vegada, l'efecte del dolor inflamatori sobre la recaiguda en el consum d'alcohol en models animals** i, per tant, constitueix una contribució important a l'estudi del dolor i l'AUD.

En conjunt, els resultats presentats en aquesta Tesi destaquen els **efectes que el dolor inflamatori** indueix sobre **el control regulador que sistema opioide endogen exerceix sobre SMCL** i, en conseqüència, l'impacte del dolor inflamatori en les **propietats reforçants de l'etanol**. D'aquesta manera, el dolor inflamatori indueix una dessensibilització dels MORs locals en el VTA, la qual cosa resulta en canvis en les accions d'etanol sobre l'activitat de les neurones de DA al SMCL. En aquest sentit, el dolor inflamatori redueix l'alliberament de DA provocat per etanol en el NAc. Una conseqüència conductual important és que el dolor redueix el CPP provocat per l'administració directa d'etanol al VTA. Finalment, en el paradigma d'exposició a l'etanol a llarg termini emprat per avaluar la influència de el dolor inflamatori en la recaiguda, els animals amb dolor desenvolupen l'ADE, encara que no s'observen canvis en la seua magnitud en comparació amb els animals control.

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