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Study of the neurobiology of ethanol-relapse behaviour and its prevention with N-acetylcysteine: preclinical studies in rats

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A Paco Flores

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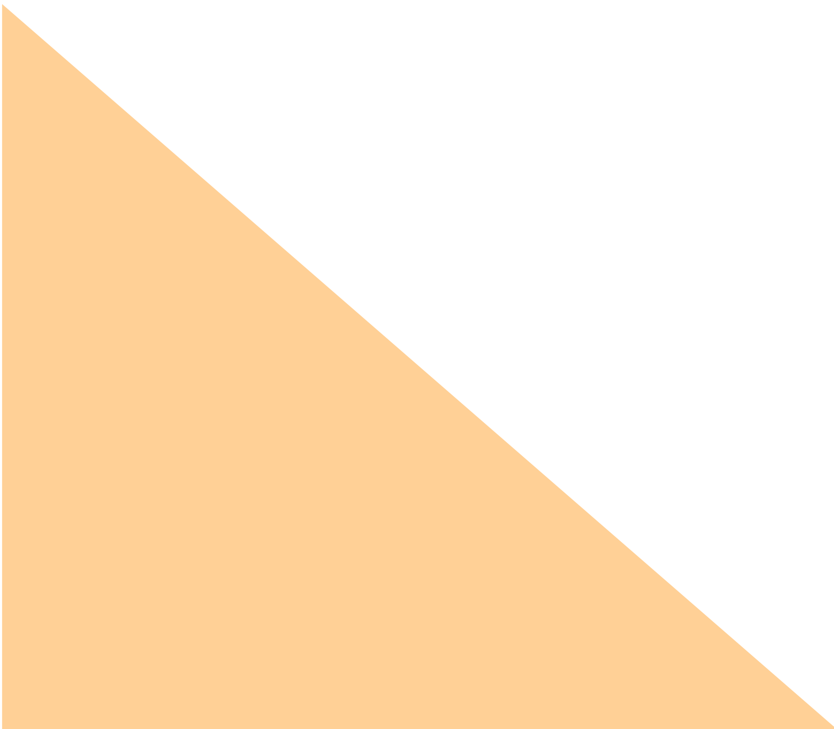
ABBREVIATIONS

ACD	Acetaldehyde
aCSF	Artificial cerebrospinal fluid
ADE	Alcohol deprivation effect
AMG	Amygdala
AUD	Alcohol use disorder
CPP	Conditioned place preference
DA	Dopamine
DS	Dorsal striatum
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporters
EMA	European Medicines Agency
FDA	Food and Drug Administration
GLAST	Glutamate aspartate transporter
GLT1	Glutamate transporter 1
GSH	Glutathione reduced
GSSG	Glutathione oxidised
HIP	Hippocampus
HMGB1	High-mobility group box 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IR cells	Immunoreactive cells

i.p.	Intraperitoneal
LTD	Long-term depression
MCLS	Mesocorticolimbic system
mGluR	Metabotropic glutamate receptor
MS	Mass spectrometry
MTEP	3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine
NAC	N-acetylcysteine
NfκB	Nuclear factor kappa B
NIAAA	National Institute on Alcohol Abuse and Alcoholism of the United States
NICE	National Institute for Health and Care Excellence
NIDA	National Institute on Drug Abuse
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
SD	Sprague-Dawley
STM	Striatum
SUD	Substance use disorder
TBS	Trizma buffer saline
TNF-α	Tumor necrosis factor alpha
UPLC	Ultraperformance liquid chromatography
VTA	Ventral tegmental area
WHO	World Health Organisation

1. INTRODUCTION



Introduction

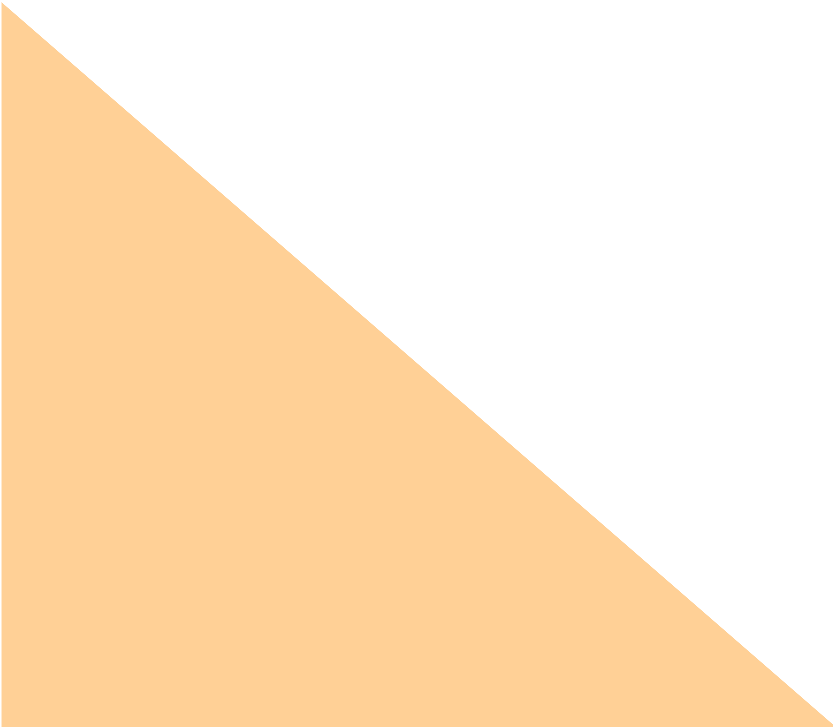
According to the World Health Organisation (WHO), alcoholism is one of the main disease burdens, being a chronic and recurring disorder that entails huge social and economic costs worldwide. Since the available pharmacotherapies for treating alcohol use disorder (AUD) provides unsatisfactory clinical outcomes, the scientific community has gone to great lengths to better understand this pathology with the purpose of improving available treatments and proposing new potential pharmacotherapies. At present, one of the most desirable goals is to prevent the overriding issue of this neurobehavioural disorder: the relapse after a period, more or less prolonged, of abstinence. Despite the efforts made by the scientific community, there are still some remaining gaps in literature, such as the study of the neuroadaptations that occur during the abstinence period. The study of this concrete period of the addiction cycle would enable the identification of potential alterations that could induce the craving that will lead to the relapse process.

Among all the neuroadaptations that potentially underlie the development of AUD, the interconnected cycle of oxidative stress, neuroinflammation and glutamate homeostasis is especially interesting. A large body of literature provides evidence about the ethanol-induced alterations in these three systems, but studies carried out during long-term abstinence are scarce. In this context, N-acetylcysteine (NAC) has been proposed as an ethanol anti-relapse treatment due to its antioxidant, anti-inflammatory and glutamatergic properties. Moreover, the preclinical efficacy of NAC on the treatment of other substance use disorders (SUDs), such as cocaine, heroin and nicotine addiction has also been demonstrated. There is a growing

interest in NAC as a treatment for AUD which is reflected in the ever-increasing number of preclinical studies focused on NAC effect on ethanol intake and relapse. Nonetheless, its mechanism of action has not yet been fully understood. Besides, its anti-relapse efficacy has not yet been proved in a high face, predictive and ecological validity model such as the alcohol deprivation effect (ADE) model. This is one of the most appropriate preclinical models to study ethanol relapse-like drinking behaviour since it encompasses the entire range of the addiction cycle, a fact that supports the model's high face and predictive validity.

The present thesis is designed to deepen the understanding of the underlying neurobiology of relapse behaviour through a three-stage study, and to evaluate the efficacy of NAC as an ethanol anti-relapse treatment by using the ADE preclinical model. Concretely, this manuscript contains experiments that allow to: (i) identify and classify animals according to their relapse-like drinking behaviour, (ii) characterise the ethanol consumption pattern of the resulting subpopulations and (iii) characterise biochemical alterations that occur in long-term abstinence. Additionally, the dose-dependent efficacy of NAC in the prevention of ethanol relapse and the mechanism underlying this effect is analysed. In particular, NAC's antioxidant, anti-inflammatory and glutamatergic properties are investigated. On top of that, a set of preliminary experiments aimed to explore the effect of NAC on glutamatergic signalling and on ethanol rewarding properties are also included.

2. STATE OF THE ART



2.1 Alcohol Use Disorder (AUD): The issue of relapses

Alcohol consumption is part of daily routine in different cultures especially in social, religious and business events. The easy access, legality and low cost of alcohol, along with the lack of prejudice against alcohol use facilitate that 43% of the world's population have consumed alcohol during the last 12 months. Although alcohol consumption is widespread among many societies, it is a psychoactive substance with toxic effects and its continuous use can result in dependence disorders (World Health Organization, 2018). From now on, the terms **alcohol** and **ethanol** are used equally in this manuscript.

According to the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013), terms like alcoholism, alcohol dependence or alcohol addiction are clinically referred to as **Alcohol Use Disorder (AUD).** The National Institute on Alcohol Abuse and Alcoholism of the United States (NIAAA) described AUD as a chronic and progressive psychopathology with **high relapse rate** characterised by the inability of the patient to stop drinking alcohol despite its negative effects (National Institute on Alcohol Abuse and Alcoholism, 2021). Although recurrent consumption of ethanol can provoke a chronic abuse pattern that can conclude in AUD, only a portion of ethanol drinkers will develop this pathology (Koob & Volkow 2010). In fact, according to the last "Global status report on alcohol and health", 5.1% of the adult population worldwide suffered from AUD and around 3 million persons had died in 2016 due to ethanol misuse (World Health Organization, 2018). Sohi and collaborators estimated the alcohol-use related premature mortality worldwide and identified tuberculosis

(190,000 deaths), road injuries (338,000 deaths) and cirrhosis of the liver (457,000 deaths) as the leading causes of the burden of alcohol-attributable premature deaths (Sohi et al., 2021)

Alcohol chronic intake is a risk factor for several diseases. In fact, AUD is frequently comorbid with psychiatric disorders such as anxiety, major depression or other substance use disorders, especially tobacco, and other pathologies such as cardiovascular and liver diseases, diabetes mellitus or cancer (Grant et al., 2015; World Health Organization, 2018). Moreover, there is a relationship between alcohol misuse and traffic accidents, aggression, homicides, sexual assaults and robbery (Galbicsek, 2021; World Health Organization, 2018). Besides the health harm caused by alcohol misuse, there is an economic burden associated with productivity loss, criminal justice system and traffic accidents (Manning et al., 2013).

The main issue of this neurobiological disorder is the **relapse**, defined as “the resumption of alcohol drinking following a prolonged period of abstinence” (Becker, 2009) (from now on the terms **abstinence** and **withdrawal** are used equally in this manuscript). For any drug, the risk of relapse among drug addict patients is quite worrisome, since it persists even many years in the absence of drug consumption (Kalivas, 2009). Regarding AUD, literature shows that **60–80% of abstinent alcoholics will relapse during their lifetime** (Barrick & Connors, 2002; Weiss et al., 2001). The guidelines for treating AUD dictated by the European Medicines Agency (EMA) point out that the goal of AUD treatments is to reach a full abstinence. However, the EMA also highlights that the reduction in alcohol consumption is a first acceptable step (European Medicines Agency, 2010). Nonetheless, as

explained below, the current available treatments for AUD show moderate rates of efficacy and low compliance rates (Ch'Ng & Lawrence, 2018) which highlights the necessity of developing new therapies to prevent relapse.

To develop these required new treatments, a deep knowledge of the neurobiological alterations that underlie AUD is essential. However, it must be considered that in the course of the disease the ethanol-induced alterations can evolve because of different scenarios such as chronic ethanol exposure, abstinence or binge drinking. **Abstinence** is a critical period of vulnerability in addiction that precedes relapse and the neuroadaptations that occur during abstinence remain poorly understood. Thus, delving into them would be crucial for improving available treatments and reducing the relapse rate or, to a lesser extent, reducing ethanol intake (Cannella et al., 2019; Reilly et al., 2014; Spanagel & Vengeliene, 2013)

Hence, the present thesis is focused on the study of these alterations. Concretely, we addressed our efforts towards the exploration of the potential alterations in glutamate homeostasis, redox status and neuroinflammatory processes in the mesocorticolimbic system (MCLS) that occurs during the abstinence period.

2.2 MESOCORTICOLIMBIC SYSTEM AND ETHANOL-INDUCED NEUROBIOLOGICAL ALTERATIONS

Despite exerting different mechanisms of action, the acute administration of any drug of abuse activates the MCLS. Additionally, chronic drug intake causes neuroadaptive changes that contribute to the development of addictive behaviours. In fact, this circuit is involved in the reward processing. The MCLS brain regions are composed of six major components (Figure 1): nucleus accumbens, prefrontal cortex (PFC), amygdala (AMG), hippocampus (HIP), striatum (STM) and ventral tegmental area (VTA) (Alasmari et al., 2018).

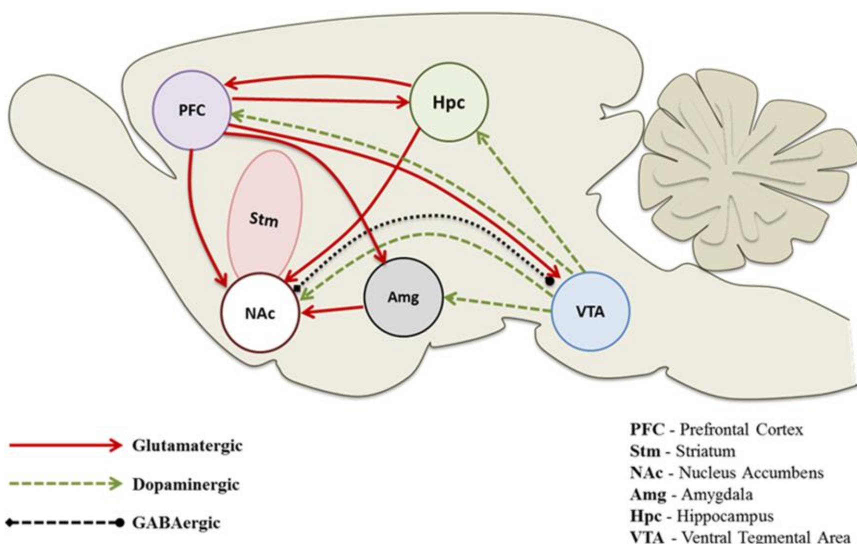


Figure 1. Scheme of the glutamatergic (red), dopaminergic (green) and GABAergic (black) connections within the MCLS. Extracted from (Alasmari et al., 2018).

Natural reinforcers such as sex, food or water consumption stimulate dopaminergic neurons of the VTA, causing dopamine (DA) release in **nucleus accumbens.** This reward mechanism leads to reinforcement behaviours that allow animal reproduction and survival. Drugs of abuse (acute intake) also cause an exacerbated stimulation of this DA circuit that, together with the reward learning, triggers the initial steps of addiction (Kalivas, 2009). Several authors suggest that after these initial steps, there is a progressive development of compulsive drug seeking and loss of control over drug intake (chronic intake) (Belin et al., 2013) that mainly depends on the ventral and dorsal STM (**DS**) as it has been observed in humans (Vollstädt-Klein et al., 2010) and in preclinical models (Belin & Everitt, 2008).

Specifically, in the case of ethanol it is known that the consumption of this drug provokes many neuroadaptations. Among them, one of the most-described in literature is the alteration of **glutamatergic homeostasis** especially in nucleus accumbens (which is explained in the following section). As shown in Figure 1, nucleus accumbens receives glutamatergic inputs from areas that are strongly related to addictive behaviours:

- **PFC** controls higher-order executive functions such as decision making. The impairment of PFC function is related to alcohol craving and relapse behaviours (Seo & Sinha, 2014).
- **AMG** is involved in the learning behaviour associated with alcohol dependence (Roberto et al., 2021).
- **HIP** controls memory formation. This process is strongly related with the alcohol-seeking behaviour that precedes relapse (Felipe et al., 2021).

Recent evidence shows that the impairment of glutamate homeostasis is linked to brain **oxidative stress** and **neuroinflammation** observed following chronic drug consumption (for extensive review see Berríos-Cárcamo et al. 2020). Thus, in this section we discuss why increasing our knowledge in the mechanisms underlying these alterations may constitute the basis for the pursuit of therapeutic strategies that could be designed for the treatment of relapse in AUD patients.

2.2.1 Glutamatergic neurotransmission

The amino acid L-glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and plays a key role in cognition, learning and memory processes. The brain has a huge concentration of glutamate which is mostly intracellular; however, there exists a continuous exchange of glutamate between cells and extracellular space (Danbolt, 2001). Most cells express glutamate receptors in their membrane (NMDA, AMPA and kainate receptors) through which glutamate exerts its excitatory effect. In order to keep the glutamatergic transmission in a physiological range, it is quite important to maintain basal extracellular glutamate concentrations. High extracellular concentrations of this neurotransmitter are toxic since immoderate activation of glutamate receptors is damaging for neurons and causes a pathological process known as **excitotoxicity** (Trotti et al., 1998).

Glutamate uptake is the main mechanism by which extracellular glutamate concentrations are kept low. The principal proteins responsible for glutamate uptake are the Na^+-dependent high-affinity glutamate transporters, also known as **excitatory amino acid**

transporters (EAAT). Five EAAT have been identified: EAAT1, also known as glutamate aspartate transporter (GLAST) in rodents; EAAT2, which is known as glutamate transporter 1 (GLT1) in rodents; EAAT3 or excitatory amino acid carrier (EAAC); EAAT4 and EAAT5. The activity of these transporters also minimises cross-activation between nearby excitatory synapses by reducing glutamate overflow from one synapse to another (Asztely et al., 1997).

GLT1 is the most abundant glutamate transporter and the major perpetrator of synaptic glutamate uptake in cerebral cortex, HIP and STM (Lehre et al., 1995). Moreover, **GLAST** is second in removing synaptic glutamate and the most expressed glutamate transporter in cerebellum (Lehre & Danbolt, 1998). Even though GLT1 and GLAST are mainly found in astrocytes, they are also detected in oligodendrocytes (DeSilva et al., 2009). Besides, some studies also reported the presence of GLT1 in presynaptic axon terminals (Danbolt et al., 2016; Furness et al., 2008). Curiously, their distribution across astrocyte membrane is unbalanced: the side of astroglia surface that faces glutamatergic synapses has higher GLT1 or GLAST protein expression than the side that is in front of a cell body, astrocytes or endothelium (Chaudhry et al., 1995) as can be seen in Figure 2 for a synapse in the HIP. Interestingly, a study found that GLT1 transporters are not static, but are instead highly mobile at the surface of astrocytes, and that this surface diffusion is dependent on both neuronal and glial cell activities (Murphy-Royal et al., 2015).

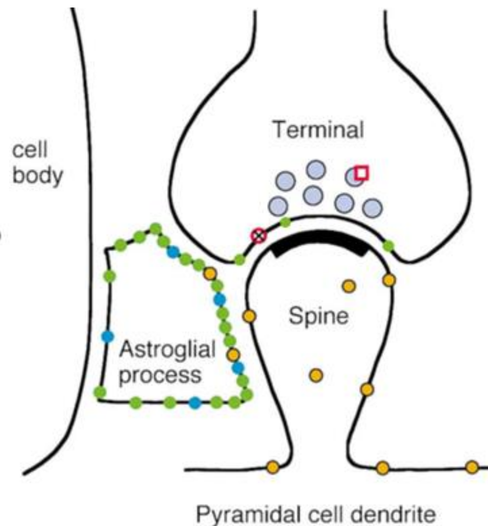


Figure 2. Schematic representation of the localisation of glutamate transporters in the vicinity of a hippocampal glutamatergic synapse. Green dots in the membranes represent GLT1, blue dots represent GLAST and yellow dots represent other glutamatergic transporters. Blue circles represent glutamate containing vesicles whereas the red square represents a vesicular glutamate transporter. Adapted from (Danbolt, 2001; 2016).

Levels of extracellular glutamate are also influenced by the activity of the anionic amino acid transporter **system xC-**. This is a cystine-glutamate antiporter composed of a light chain (xCT) and a heavy chain (4F2) (Sato et al., 1999) that works in a Na+ independent way. This protein is mainly expressed in astrocytes and microglia, but also in neurons. It interchanges intracellular glutamate for extracellular cystine in a 1:1 ratio (Massie et al., 2015) and works as the major extracellular glutamate supplier in many brain regions such as nucleus accumbens, STM or HIP (Baker et al., 2002; de Bundel et al., 2011). Thus, system xC- activity has two immediate consequences. On the one hand, it provides cysteine for **glutathione** (GSH) synthesis in the astrocyte

(detailed in the next section). On the other hand, it augments extrasynaptic glutamate levels which triggers the activation of metabotropic glutamate receptors (mGluR).

mGluR are a family of G-protein coupled glutamate receptors which is divided into different groups regarding their activation and coupling: group I comprises mGluR1 and mGluR5, group II includes mGluR2 and mGluR3 and group III encompasses mGluR4, mGluR6, mGluR7 and mGluR8 (Danbolt, 2001). The present thesis gives special attention to **mGluR2** (expressed predominantly in olfactory bulb and cerebellar cortex); **mGluR3** (extensively expressed in cerebral cortex, nucleus accumbens, STM and cerebellar cortex); and **mGluR5** (mainly located in the cerebral cortex, HIP, nucleus accumbens, and STM) (Crupi et al., 2019). Hereinafter, mGluR2 and mGluR3 will be referred to as **mGluR2/3 protein.**

mGluR2/3 are presynaptically located and, when activated, have an inhibitory role in glutamatergic synapse reducing the probability of glutamate release. On the contrary, mGluR5 has an excitatory role in glutamatergic synapse and it is placed in the postsynaptic neuron. Apparently, mGluR2/3 has a higher affinity for glutamate than mGluR5, thereby the augmentation of extracellular glutamate levels has an inhibitory effect on glutamatergic synapse. However, if this augmentation is exacerbated, the low affinity mGluR5 receptor will be activated, causing the opposite effect (Kupchik et al. 2012).

To illustrate this information, Figure 3 represents a glutamatergic synapse in nucleus accumbens where the above-mentioned proteins are represented. The figure also includes the values of synaptic, extrasynaptic and astroglial glutamate concentrations.

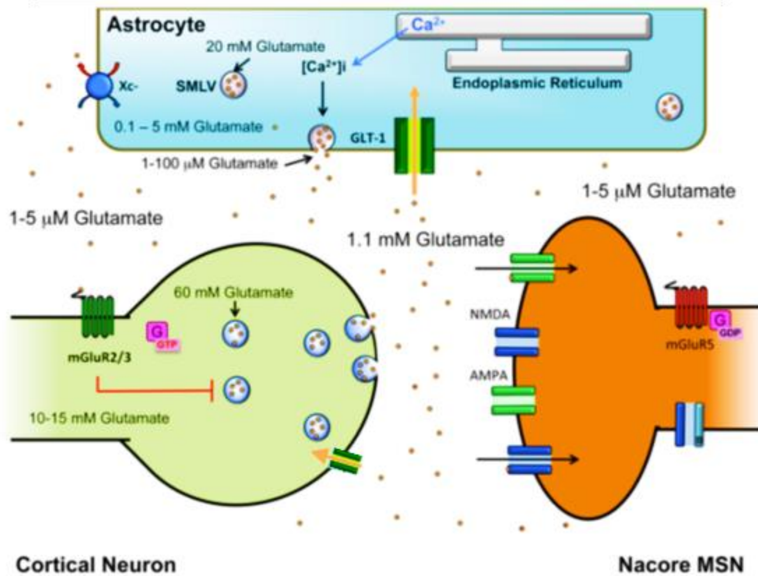


Figure 3. Schematic glutamatergic synapse in the nucleus accumbens with the pre- (green) and postsynaptic (orange) neurons as well as an astrocyte (blue). Glutamate is represented as orange spheres and concentrations of glutamate are also shown. As depicted, glutamate tone activates presynaptic mGluR2/3, which inhibits the release of glutamate (red line). System xC- and GLT1 are shown releasing and taking up glutamate, respectively. On the postsynaptic side mGluR5 receptors are shown as well as other glutamatergic receptors: AMPA and NMDA receptors. MSN: Medium spiny neurons. SMLV: synaptic-like microvesicle. Adapted from (Scoefield & Kalivas, 2014).

Next, we are going to discuss how these proteins and the glutamate homeostasis are altered by ethanol consumption and abstinence.

ALTERATIONS IN GLUTAMATE HOMEOSTASIS CAUSED BY ALCOHOL MISUSE

According to the literature, acute ethanol consumption alters the balance between excitatory and inhibitory neurotransmission in the brain by diminishing the excitatory and strengthening the inhibitory neurotransmission. When ethanol consumption is prolonged, the brain

responds to this alteration of neurotransmission by enhancing the excitatory and reducing inhibitory (Rao et al., 2015a). The maintenance of exacerbated excitatory neurotransmission is linked to ethanol tolerance and dependence (Littleton, 1995). When ethanol consumption is ceased **ethanol withdrawal syndrome** appears, which is a hallmark of AUD. It is characterised by the manifestation of unpleasant symptoms, some of which caused by an augmented glutamatergic transmission, such as anxiety, seizures or irritability (Roberto et al., 2012). This increase in glutamatergic transmission during abstinence is reported to be critical for the subsequent ethanol **relapse** (Alasmari et al., 2018).

Accumulating evidence suggests that a common mechanism that could underlie the relapse process of different drugs of abuse involves the above-mentioned glutamatergic projections from PFC, HIP and AMG to nucleus accumbens (Figure 1). According to the so-called glutamate hypothesis of drug addiction proposed by Dr. Kalivas (Figure 4), the presence of contextual cues associated with drug consumption is processed by these areas and finally causes an exacerbated glutamate release in nucleus accumbens that triggers drug-seeking behaviours and the subsequent relapse (Kalivas, 2009). In fact, glutamate homeostasis in nucleus accumbens seems to be involved in the vulnerability to relapse to drug-taking (Kalivas & Volkow 2005).

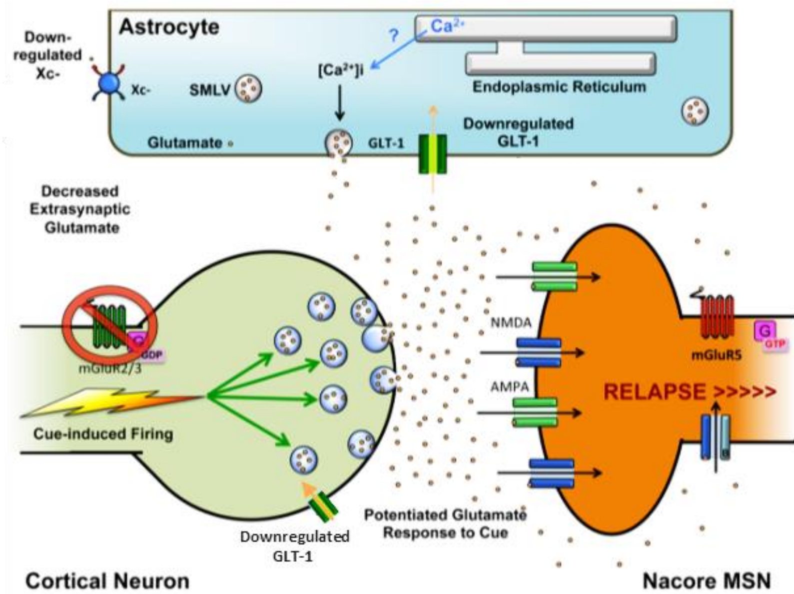


Figure 4. Glutamatergic synapse in nucleus accumbens during the abstinence period. In the picture, the pre- (green) and postsynaptic (orange) neurons as well as an astrocyte (blue). Glutamate is represented as orange spheres. As hypothesised, GLT1 is downregulated during abstinence and synaptic glutamate mostly activates the postsynaptic neuron. According to this hypothesis, system xC- is also downregulated, so there is not enough extracellular glutamate to activate mGluR2/3. This situation potentiates glutamate release leading to spillover and the activation of postsynaptic glutamate receptors, which promotes drug-seeking and the subsequent relapse. MSN: Medium spiny neurons. SMLV: synaptic-like microvesicle. Adapted from (Scofield and Kalivas 2014).

The glutamate homeostasis hypothesis of drug addiction (Figure 4), which is mainly based on cocaine research, claims that the expression of system xC- in nucleus accumbens is downregulated after chronic cocaine consumption which entails reduced levels of extrasynaptic glutamate. These reduced levels are not able to activate the inhibitory mGluR2/3 presynaptic receptor and, consequently, the synaptic

release of glutamate is increased not only after cocaine administration, but also during the **cue-, stress- or drug-induced reinstatement** of cocaine seeking, when glutamatergic inputs from PFC are stimulated. Moreover, according to this hypothesis GLT1 is downregulated after cocaine self-administration. Thus, the exacerbated levels of glutamate during reinstatement would be caused not only by the augmented release of synaptic glutamate but also by the reduction in glutamate uptake (Kalivas, 2009).

Regarding ethanol, human studies are in accordance, at least in part, with this hypothesis. In this line, augmented glutamate plasma levels were detected in AUD patients 12 hours after alcohol exposure (Brousse et al. 2012). In this sense, many efforts have been focused on unravelling the relationship between ethanol misuse and glutamate homeostasis. Consequently, several preclinical studies have explored the effects of alcohol exposure on the extracellular glutamate levels in nucleus accumbens. The alterations on the above-mentioned glutamatergic proteins such as GLT1, GLAST and xCT (the catalytic subunit of system xC-) have also been analysed. Table 1 summarises some of the reported results, which are presented considering the paradigm of alcohol exposure and the rat strain used.

Alcohol exposure	Rats	Glutamate	GLT1	GLAST	xCT	Reference
Chronic EC	♀ P	⇧	=	⇩	=	Ding, 2013
Chronic EC	♂ P		⇩			Sari, 2013
Chronic EC	♂ P		⇩	=		Alhaddad, 2014
Chronic EC	♂ P	⇧	⇩			Das, 2015
Chronic EC	♂ P		⇩	=	⇩	Hakami, 2016
Chronic EC	♀ UChB		=	=	=	Ezquer, 2019
Intermitent EC	♂ SD	⇧	=		=	Pati, 2016
Intermitent EC	♂ SD		=		=	Stennet, 2017
8 hours abstinence	♂ Wistar		=		⇩	Lebourgeois, 2019
12 hours abstinence	♂ Wistar				⇩	Peana, 2014
16 hours abstinence	♂ Wistar	⇧				Saellstroem Baum, 2006
24 hours after 7 days i.p.	♂ SD	⇧	=	=		Melendez, 2005
Two-weeks abstinence	♀ P	=	=	⇩	=	Ding, 2013
Reinstatement	♂ P		⇩	=	⇩	Hammad, 2021 *
3 days reaccess	♂ UChB		=		=	Ezquer 2022
7 days reaccess	♂ P		=		=	Das, 2022

*Table 1. Alterations in extracellular glutamate levels and expression of GLT1, GLAST and xCT determined in the rat nucleus accumbens under different experimental conditions. If the research did not report any data the box is empty. EC = ethanol consumption; i.p. = intraperitoneal; P = P rat strain; UChB= UChB rat strain; SD = Sprague-Dawley rat strain. * The article of Hammad 2021 reported downregulation of GLT1 and xCT expression in nucleus accumbens shell but not core*

Overall, the results showed in the table seem to evidence that:

- **Extracellular glutamate levels** in nucleus accumbens increases after chronic or intermittent ethanol consumption but also during early-abstinence. Nonetheless, after two weeks of alcohol abstinence glutamate levels seem to be restored.
- **Chronic ethanol consumption** diminished GLT1 expression in male but not in female animals. However, the experiments conducted in **female** rats are scarce.
- **Chronic ethanol consumption** mainly reduces or does not alter GLAST and xCT expression in nucleus accumbens.
- Apparently, early abstinence seems to reduce xCT expression in male animals. However, there is a lack of studies focused on the protracted **abstinence period** and ethanol **reintroduction**.

As can be deduced from the above observations, the results obtained through the study of the transporters are not fully coincident. Nonetheless, there is a general agreement about the augmented extracellular glutamate levels in the different experimental conditions. This scenario would cause the overactivation of postsynaptic neuron causing **excitotoxicity**. An immediate consequence of this hyperexcitatory environment should be the increase in reactive oxygen species (ROS) and neuronal damage (Galduróz et al., 2015). The consequences of augmented ROS levels and their interplay with ethanol consumption and abstinence, are analysed in the next section.

2.2.2 Oxidative stress

ROS are small molecules, such as superoxide anions or hydrogen peroxide, that are extremely reactive and can irreversibly alter proteins, lipids and DNA (Brieger et al., 2012). Under physiological conditions ROS are produced as a result of aerobic metabolism and play a role in cellular processes such as proliferation, survival or apoptosis. When an exacerbated production of ROS that cannot be counterbalanced by antioxidant defences is generated, the phenomenon of **oxidative stress** ensues (Womersley & Uys, 2016). This pathophysiological condition defaces cellular organelles and can cause cell death (Ye et al., 2015).

Brain functioning requires a considerable quantity of oxygen, whose metabolisation produces high ROS levels. Due to the low prevalence of antioxidant enzymes and the presence of polyunsaturated lipids, the antioxidant defences are crucial to maintain redox homeostasis in the brain (Dringen, 2000). The major cellular antioxidant is a γ-glutamyl-cysteine-glycine tripeptide known as **glutathione (GSH)**, which exists in brain tissues at a concentration of 2-3 mM. In an oxidant environment, two molecules of GSH (reduced) are oxidised and combined to form the glutathione dimmer i.e., the oxidised molecule (**GSSG**) (Figure 5 A). This reaction neutralises ROS and maintains redox homeostasis. **The ratio GSSG/GSH provides an indication of the redox status and it is extensively used as an oxidative stress marker** (Aoyama, 2021).

GSH content in neurons is lower than in astrocytes (Dringen et al., 1999). In fact, neurons need the support of astrocytes to maintain GSH synthesis. As previously explained, the antiporter system xC- introduces extracellular cystine into the astrocyte where it is reduced to two

molecules of cysteine that serve as a substrate for GSH synthesis (Figure 5 B) (Berríos-Cárcamo et al., 2020).

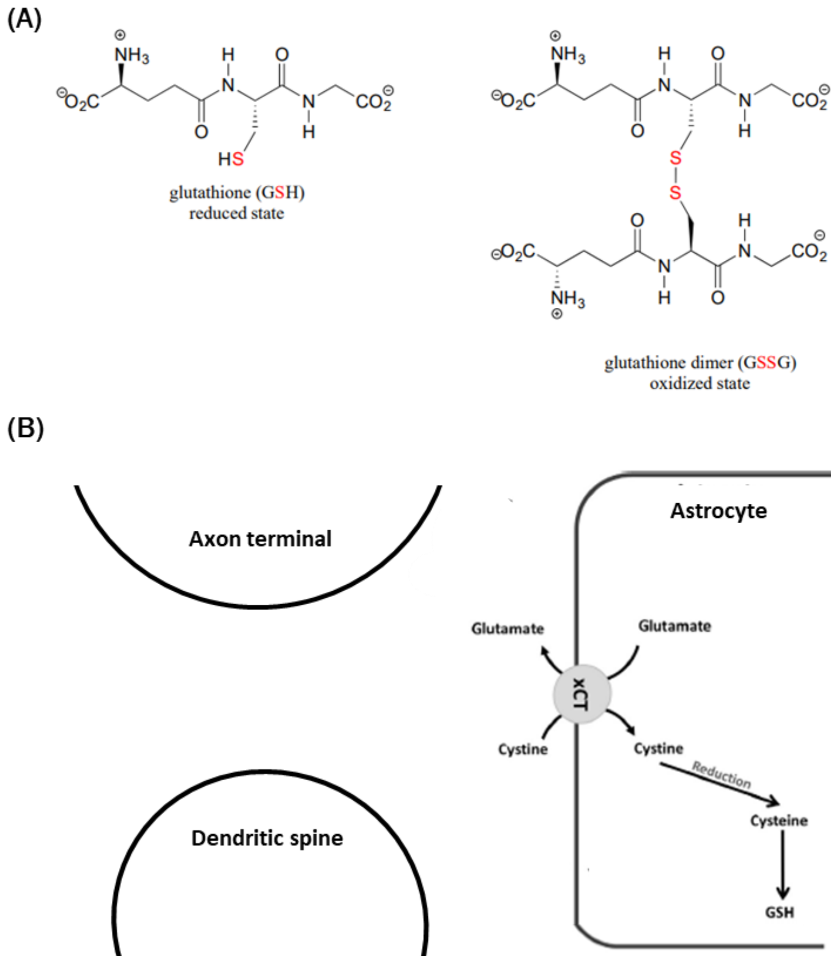


Figure 5. (A) Structure of glutathione. On the left it is shown in its reduced form (GSH) and on the right the oxidised molecule dimer (GSSG) is displayed. Sulphide is shown in red. Extracted from (Soldelberg, 2022). (B) Schematic representation of system xC (xCT) activity. The antiporter interchanges intracellular glutamate for extracellular cystine. Once in the astrocyte, cystine is reduced to cysteine, that acts as a substrate for GSH synthesis. Adapted from (Womersley et al., 2018).

The role of system xC- in GSH synthesis implies an **interaction between glutamate homeostasis and redox status**. Besides, in a prooxidant environment the activity of the antiporter is diminished causing a reduction in astrocytic cysteine content (Berríos-Cárcamo et al., 2020). A prooxidant condition also inhibits the activity of GLT1 and GLAST (Trotti et al., 1988). Under these circumstances, there is an excess of extracellular glutamate that also inhibits the activity of system xC-. Thus, astrocytes do not receive a sufficient amount of cysteine and the synthesis of GSH is reduced (Lewerenz et al., 2006).

The prooxidant condition that affects glutamate homeostasis and GSH synthesis could be a consequence of ethanol consumption and abstinence as explained below.

ALCOHOL MISUSE CAUSES AN IMBALANCE IN BRAIN REDOX STATUS

Antioxidants are quite important in brain areas innervated by **dopaminergic** neurons, such as nucleus accumbens, HIP, AMG or PFC (Figure 1) because DA metabolism results in increased ROS production. It is well known that consumption of drugs of abuse stimulates dopaminergic transmission causing an augmentation of DA metabolism with a subsequent increase in ROS levels. Moreover, drugs of abuse are also able to stimulate ROS production through an additional process: the auto-oxidation of dopamine (Womersley et al., 2019).

In the case of ethanol, additionally, its metabolism is related with increased ROS production. In fact, ethanol is metabolised into

acetaldehyde (ACD) in a process that produces hydroxyl and hydroxyethyl radicals. The accumulation of ACD is associated with augmented oxidative stress which damages cells and organelles, especially the mitochondria leading to apoptotic events (Hernández et al., 2016; Tapia-Rojas et al., 2018).

All in all, substantial evidence associates **chronic ethanol consumption** with oxidative stress in the brain. For instance, preclinical studies have reported that after ten weeks of ethanol exposure rats displayed lower GSH levels in HIP and cortex of male Wistar rats (Tiwari & Chopra, 2013). Reddy and collaborators found, after sixty days of forced oral ethanol administration, altered mitochondrial membrane properties and augmented TBARS and protein carbonyl content in the cortex of male Wistar rats (Reddy et al., 2013). Furthermore, it has also been reported that voluntary chronic ethanol consumption reduced thiols in plasma and increased oxidative stress levels in the HIP of female UChB rats (Quintanilla et al., 2018).

During the **ethanol deprivation** period an imbalance in oxidative status was also detected. The augmented glutamate levels associated with the abstinence period could induce an overactivation of the postsynaptic neuron leading to a huge calcium influx. This influx could increase the nitric oxide synthase enzyme activity and alter the mitochondrial membrane producing great amounts of ROS (Jung, 2015). Apparently, the toxic effects associated with ethanol consumption and/or ethanol abstinence are different. Hence, differences in the brain damage they cause have also been identified (Jung & Metzger, 2010). In this sense, Elibol-Can and collaborators revealed several profound changes in the content, structure, and

function of lipids, proteins, and nucleic acids taking place in the **HIP** of male Wistar rats after ethanol exposure. These authors reported that 72 hours after ethanol withdrawal some alterations ameliorated, but the changes in lipids were aggravated, indicating development of a withdrawal-induced oxidative stress. They suggested that the oxidative damage associated with the early ethanol withdrawal period could be more harmful than the damage linked to previous ethanol consumption (Elibol-Can et al., 2011). In agreement with their findings, other authors reported that early ethanol abstinence produced a greater lipid peroxidation and protein oxidation than ethanol *per se* in female Sprague-Dawley rats (Jung et al., 2004, 2008). On the other hand, Jung and Metzger conducted an experiment by which they demonstrated that there were differences in the ethanol abstinence-induced oxidative damage between male and female Sprague-Dawley rats after three weeks of abstinence (Jung & Metzger 2016). To our knowledge, this is the only study that explored the oxidative stress status after protracted abstinence. Hence, exploring **the redox brain status after a prolonged ethanol abstinence period could help us to delve into the neurobiology of relapse and the associated craving process.**

To conclude this section, it must be highlighted that neuroinflammatory processes can also be linked to glutamate neurotransmission and brain redox status. Concretely, augmented ROS levels activate astrocytes and microglia stimulating the release of proinflammatory factors that would enhance oxidative stress (Berríos-Cárcamo et al., 2020). Hence, the neuroinflammatory response associated is briefly summarised in the next section.

2.2.3 Neuroinflammation

The brain is somewhat isolated from systemic immune cells and peripheral signals due to the existence of the so-called blood-brain barrier (Louveau et al., 2015). Consequently, the neuroinflammatory response is mainly mediated by **microglia** and **astrocytes,** the principal brain immune cells (Colombo & Farina, 2016; Kraft & Harry, 2011). Pathological changes stimulate the activation of the microglial pathogen recognition receptors, such as the toll-like receptor-4 (TLR-4). The activation of this receptor induces the transcription of pro-inflammatory factors through the **nuclear factor kappa B (NfκB)** pathway that leads to the transcription **of interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) or inducible nitric oxide synthase (iNOS)** (Fernandes et al., 2017). On the other hand, the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) can also be activated (then called inflammasome) stimulating IL-1β secretion. One of the most known is **NLRP3** which is a sensor of metabolic stress and is associated with neuroinflammatory and demyelination processes that underlies the development of different neurodegenerative diseases (Kelley et al., 2019). The released proinflammatory factors will amplify the neuroimmune response through the stimulation of TLR4 and cytokine receptors of microglial cells, astrocytes, oligodendrocytes and neurons (Blanco & Guerri, 2007). The purpose of this signalling cascade is to contribute to tissue repair, neurotrophic factors release, matrix remodelling and cellular debris removal (Harry & Kraft, 2008).

One of the pathological conditions that can trigger neuroinflammatory cascades is oxidative stress. ROS can directly activate the transcription of NfκB which enhances the synthesis of pro-oxidant enzymes (such as iNOS) and pro-inflammatory molecules (such as IL-1β and TNF-α). As commented above, drugs of abuse induce DA release, the metabolism of which increments oxidative stress levels and the subsequent production of inflammatory mediators. Pro-inflammatory cytokines in turn activate microglia and astrocytes, increasing the production of oxidative stress which will perpetuate the cycle. Noteworthily, oxidative stress and inflammation further self-potentiate each other, as can be observed in Figure 6 (Berríos-Cárcamo et al., 2020). On the other hand, drugs of abuse *per se* can also promote NfκB activity through TLR4 receptors, as demonstrated for cocaine (Northcutt et al., 2015), opioids (Eidson et al., 2017; Hutchinson et al., 2010) and ethanol (Crews et al. 2013; Fernandez-Lizarbe et al., 2009; Zou and Crews 2014).

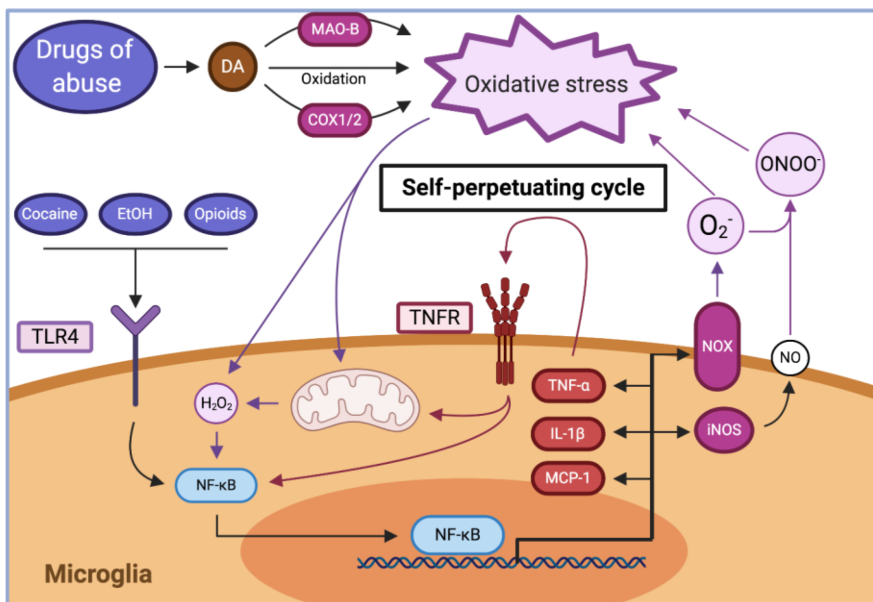


Figure 6. Self-perpetuating cycle of oxidative stress and neuroinflammation in the context of drugs of abuse consumption. Drugs of abuse increase DA release, the metabolism of which enhances oxidative stress levels. This prooxidant condition stimulates NfκB activity in microglia and the production of neuroinflammatory mediators and prooxidant enzymes (NADPH-oxidase (NOX) and iNOS) which, in turn, augments oxidative stress. Cocaine, ethanol (EtOH) and opioids can directly promote NfκB activity through the activation of TLR4 receptors. MAO-B: monoamine oxidase B; COX 1/2: cyclooxygenase 1/2 (COX1/2); MCP-1: monocyte chemoattractant protein-1. Figure obtained from (Berríos-Cárcamo et al., 2020)..

Likewise, a crosstalk between glutamate levels and immunomodulatory signals has also been described in literature. Concretely, changes in brain glutamate levels can cause microglial liberation of proinflammatory factors (Hagino et al., 2004; Murugan et al., 2011). Furthermore, immunomodulatory signals can alter synaptic transmission through its influence over astrocytes (Cekanaviciute & Buckwalter, 2016). An example of this interaction is displayed by the proinflammatory factor **high-mobility group box 1 (HMGB1)** which is released by excited neurons and glia and is recognised by microglial TLR4. This factor activates microglial cells which, in turn, release proinflammatory factors that affect astrocytes. Due to this signalling, astrocytes reduce glutamate uptake and increase glutamate release, thereby increasing neuronal excitability which supports the release of additional HMGB1 (Crews & Vetreno, 2016). Specifically, one proinflammatory factor that shows this effect on astrocytes is TNF-α, which reduces the expression of GLT1 and GLAST in astrocytes (see review Gipson et al., 2021).

As previously mentioned, drugs of abuse can directly activate TLR4 receptors. The possible mechanism by which ethanol triggers

inflammatory response in the brain and the alterations in neuroinflammatory mediators observed during the different phases of AUD are explained below.

Neuroinflammatory processes as a consequence of ethanol misuse

It is suggested in the literature that in ethanol abuse, the metabolism of ethanol in the gut provokes an augmentation of peripheral inflammatory processes that finally enhances neuroinflammatory signals in the brain (Berríos-Cárcamo et al., 2020). In particular, systemic TNF-α can stimulate a neuroinflammatory cascade through the activation of brain TNF-α receptors as well as the activation of the blood brain barrier endothelial cells and pericytes (Banks, 2015; Qin et al., 2007). Even so, neuroimmune response is also triggered by neuronal damage caused by ethanol *per se* (Erickson et al., 2019).

Accumulating evidence suggests that **ethanol exposure triggers neuroinflammatory cascades**: an *in vitro* study, conducted in rat and mice microglial cell culture, demonstrated that acute ethanol presence caused microglial activation through its interaction with TLR4, leading to a subsequent augmentation of pro-inflammatory molecules such as TNF-α, IL-1β, iNOS or COX2 (Fernandez-Lizarbe et al., 2009). Equally, in astrocytes culture, 24 hours of ethanol exposure was able to activate NLRP3 inflammasome leading to IL-1β release (Alfonso-Loeches et al., 2014). In the same line, different *in vivo* studies performed in rodents reported that chronic ethanol exposure activated immune processes in the brain and serum (Pascual et al. 2015; Qin et al. 2008). Literature suggests that the **cortex** is a brain area critically affected by ethanol-induced neuroinflammation. Concretely, a study performed in post-

mortem human brains showed activated microglial cells in the cortex of AUD patients (He & Crews, 2009). Similarly, Sanchez-Alavez and collaborators demonstrated that 5 weeks of ethanol vapour exposure augmented the expression of Iba-1, a marker of microglial activation, in the frontal cortex and other brain areas of Wistar rats. Surprisingly, they also reported no change in the cytokine profile in frontal cortex tissue (Sanchez-Alavez et al., 2019).

Regarding the **abstinence** period, Sanchez-Alavez and collaborators reported that the augmented expression of Iba-1 remained elevated after 28 days of abstinence, whereas neither 1 nor 28 days of ethanol abstinence changed the cytokine profile in the frontal cortex of Wistar rats (Sanchez-Alavez et al., 2019). Furthermore, it has also been shown that intermittent ethanol exposure caused neurobehavioural dysfunction, neuronal damage, microglial activation and increased cytokines expression in cortex and HIP of Sprague-Dawley rats. However, the same study also revealed that these alterations tended to be restored after 4 days of abstinence (Zhao et al., 2013). Other preclinical studies did detect augmented neuroinflammatory mediators during the ethanol withdrawal period: a study performed in rats reported that the enhanced expression of cytokine mRNA within 29 hours of ethanol withdrawal was brain area dependent, with the cortex being the most affected region (Knapp et al., 2016). Other authors, using adolescent rats, found increased levels of TNF-α in the frontal cortex 25 days after intermittent alcohol exposure (Vetreno et al., 2013). In a clinical study, alcoholic patients presented higher serum levels of inflammatory markers such as TNF-α, IL-1β or IL-6 after their last alcohol consumption, but these levels were diminished after

withdrawal (Girard et al., 2019). All in all, the data reported hitherto do not allow us to draw firm conclusions about how inflammatory processes evolve during the ethanol abstinence period.

Consequently, literature claims the necessity of further studies to explore how neuroinflammation is altered during the different stages of the addiction cycle (Gipson et al., 2021). Thus, the latest review of Namba and collaborators pointed out the lack of studies comparing how long-term abstinence and previous drug exposure can modify neuroinflammatory processes. They suggest the importance of **understanding whether these alterations during protracted abstinence could be related with the incubation of the craving and the subsequent drug-seeking behaviour.** They also claim that since the neuroinflammatory response related to AUD depends on the duration of ethanol consumption and withdrawal periods, both factors must be taken into consideration to fully understand how ethanol affects immunomodulatory signals (Namba et al., 2021).

In our opinion, the use of a suitable preclinical model that mimics the human addiction pattern and allows us to analyse biochemical alterations through the different stages of the addiction cycle would provide the most translational and valuable results.

2.3 Preclinical models to study alcohol relapse-like behaviour

Drug addiction is a complex human mental disorder. Hence, modelling this neuropsychiatric disorder in rodents is a challenge given its complexity (Kuhn et al., 2019). With this in mind, different animal models have been designed to study this disorder. These preclinical models should mimic human addictive behavioural traits to provide a high degree of **face validity**. Besides, the neurochemical and neuroanatomical substrates involved in the addiction process should be similar in laboratory animals and humans. This gives **construct validity** to the experimental model and turns it into a useful tool for understanding the biological mechanisms that underlie AUD. Lastly, preclinical models should have been proven to be helpful to propose therapeutic targets for new pharmacological treatments, which is known as **predictive validity** (Sanchis-Segura & Spanagel, 2006; Spanagel, 2017).

The selection of experimental paradigms is quite important since the biochemical alterations caused by ethanol are influenced by different variables such as the administration mode e.g. ethanol self-administration vs. experimenter-administration (Namba et al., 2018). Besides, a comparative study carried out by Vangeliene and collaborators reported that different rat strains differentially responded to ethanol deprivation and stress, showing that the genetic background of these rat strains affected relapse-like drinking and stress-induced drinking (Vengeliene et al., 2003). Hence, **the selection of a rat strain is also highly relevant.**

As the main goal in the treatment of AUD is the reduction of the relapse rate in abstinent patients, preclinical studies are focused on the development of new anti-relapse pharmacotherapies. These studies often use **animal models of relapse or reinstatement** in which tests are performed after a period of abstinence (forced, voluntary or extinction training) (Amaral et al. 2021; Vengeliene et al., 2014). **Reinstatement models** are operant self-administration models in which the animal, during a first phase called conditioning, learns to perform a certain task, such as manipulating what is called an operand (for example, pressing a lever) to obtain the drug. In this learning or conditioning phase, the manipulation of the operand is associated with the obtention of the drug but is also often associated with the presentation of a cue or visual stimulus (for example, a light) or sound (a certain sound of a specific frequency). After reaching stable ethanol consumption, due to the stable manipulation of the operand, animals are subjected to an extinction phase. During this period the manipulation of the operand is no longer associated with drug availability and, consequently, the animal stops performing the task. After extinction, the experimenter places the animal in different situations and/or stimuli that had previously been paired with the drug experience during the conditioning phase. The researcher can evaluate whether the animal reinstates the operand manipulation response even without the presence of the drug: this is the so-called reinstatement phase. Three types of situations or stimuli are normally used to trigger drug-seeking behaviour: cues or environmental stimuli (which had previously been associated with the reinforcing effects of the drug), the administration of a non-contingent small dose of the drug (priming effect), or situations of stress (Shaham et al., 2003). The

advantage of these procedures is that the experimenter can vary the complexity of the response that the animal must perform to obtain the drug. Hence, in the context of ethanol research, the animal's degree of motivation to seek alcohol can be evaluated (Samson & Doyle, 1985). There are many authors who consider reinstatement patterns to be a good indicator of drug seeking and relapse to alcohol use (Rodd et al., 2004; Spanagel & Kiefer, 2008). In this sense, currently the most widely used anti-relapse drugs for the treatment of alcoholism, acamprosate and naltrexone, are capable of suppressing reinstatement after an extinction phase (Bachteler et al., 2005; Katner et al., 1999). This observation justifies the predictive validity of this preclinical model.

However, **relapse** can be defined as a return to alcohol consumption after a period of abstinence. Therefore, other authors consider reinstatement a valid model for the study of drug-seeking behaviour but not for the study of relapse, since:

- There is not an actual abstinence period (the animal continues to perform the task to obtain the drug, even if it does not receive it).
- The animal does not receive the drug during the reinstatement phase, so the psychoactive effects associated with its intake do not occur (Sanchis-Segura & Spanagel, 2006; Shaham et al., 2003).

For these reasons, this reinstatement model lacks construct validity: its resemblance of human behaviour is limited. As far as we are concerned, there is another preclinical model of alcohol relapse, based on the so-called **alcohol deprivation effect, the ADE model**, which is described and discussed in the next section.

2.3.1 The preclinical model based on the assessment of the alcohol deprivation effect

The preclinical model based on the manifestation of ADE, is a preclinical paradigm that mimics human relapse-like behaviour. It was described for the first time by Sinclair and Senter: animals are allowed to voluntarily drink ethanol during a period of time and then ethanol is removed during several days. When ethanol is reintroduced, animals increase ethanol intake during a limited period of time in comparison to the amount of ethanol that they used to consume before experiencing the abstinence period (Sinclair & Senter, 1968). This phenomenon is known as **ADE manifestation**, and it is defined as a significant and transient increase in ethanol intake after a period of forced deprivation. As ethanol is a weak reinforcer, this model requires **long-term ethanol exposure** with repeated deprivation periods to induce compulsiveness during a relapse situation (Spanagel, 2017). High ethanol consumption rates during basal values avoid the manifestation of ADE (Fredriksson et al. 2015, 2023). Conversely, the robustness of ADE manifestation can be augmented by:

- The simultaneous access to different alcohol concentrations (such as 5%, 10% and 20% v/v ethanol solutions in water) (Spanagel & Hölter, 1999).
- The duration of ethanol consumption: a minimum of 6 to 8 weeks of ethanol consumption is needed to manifest a steady ADE (Wolffgramm & Heyne, 1995).
- The duration of the abstinence period: a minimum of two days is needed to display ADE behaviour (Sinclair et al., 1973).

Since it was first described, ADE has been observed not only in rats (Rodd et al. 2004; Sinclair and Tiihonen 1988) but also in mice (Salimov & Salimova, 1993), monkeys (Kornet et al., 1991) and in humans (Burish et al., 1981). Furthermore, the ADE phenomenon has been identified under non-operant continuous voluntary alcohol drinking procedures, under intermittent procedures, as well as under operant self-administration paradigms (Martin-Fardon & Weiss, 2013; Martí-Prats et al., 2015a). Under the ADE preclinical model animals display different behaviours that would allow us to classify them as more or less vulnerable animals, which reflects the heterogeneity found in human patients (Spanagel, 2017). The ADE model encompasses the entire range of the addiction cycle including acquisition and maintenance of drug taking, withdrawal and craving during periods of drug abstinence and ultimately relapse, processes that are repeated several times in this experimental model. All these aspects justify the model's **high face validity** (Bell et al., 2017; Leong et al., 2018). The **predictive value** of this experimental approach is supported by the fact that current treatments for human AUD patients, such as naltrexone and acamprosate, also reduced the manifestation of the ADE phenomenon (Bachteler et al., 2005; Hölter & Spanagel, 1999). All these characteristics justify its use in the development of new anti-relapse treatments (Orrico et al. 2014; Spanagel et al. 2014; Vengeliene et al., 2007). All in all, this ADE-based model is probably one of the most commonly used preclinical approaches to study the ethanol relapse-like drinking behaviour, due to its face, predictive and ecological validity (Spanagel, 2017).

One of the few drawbacks of this animal model is the fact that the ethanol abstinence and re-access is forced by the experimenter, i.e., it is not controlled by the animal which does not allow to study the motivation to ethanol-seeking previous to relapse (Lê & Shaham, 2002). This is the reason why new preclinical models that incorporate voluntary abstinence are being developed. Nevertheless, at present, it is not known whether the use of these novel models will improve the predictive validity of classical relapse models as, to date, there are no published reports showing their postdictive validity using approved medications (Fredriksson et al., 2021).

All in all, the ADE-based model has become a widely used paradigm for studying the efficacy of new drugs that could potentially be useful in the relapse prevention in alcohol consumption. Several reviews show that processes underlying the relapse phenomenon seem to include the same neuronal circuits in rodents and humans, making animal models an excellent basis for the development of new pharmacotherapies (Bell et al., 2017; Spanagel, 2017). Therefore, the results achieved in pre-clinical studies that use ADE evaluation provide a solid basis for the subsequent development of controlled clinical trials.

2.4 Pharmacotherapies for AUD treatment

Notwithstanding the socioeconomic and health harm caused by AUD, a low percentage of patients receive adequate treatment. In this context, a very recent study reported that only 1.6% of adults in the USA with past-year AUD were treated with evidence-based AUD pharmacotherapies (Han et al., 2021). These data clearly highlight the importance of developing more effective treatment strategies. Hitherto, apart from the psychosocial interventions that are crucial for AUD patient recovery, only five drugs are approved for the treatment of AUD.

2.4.1 Approved pharmacotherapies for preventing alcohol relapse

The first approved pharmacotherapy for AUD was **disulfiram** (Food and Drug Administration, FDA, 1951) which is an inhibitor of the ACD dehydrogenase. During the metabolism of ethanol in the liver, the alcohol dehydrogenase oxidises alcohol into ACD which is converted into acetate by the action of ACD dehydrogenase. The inhibition of this last enzyme by disulfiram causes an accumulation of peripheral ACD which is responsible for aversive symptoms such as nausea, tachycardia, hypotension, or vomits (Kranzler & Soyka, 2018; Witkiewitz et al., 2019). Thus, patients must remain abstinent so as to avoid experiencing this whole set of unpleasant effects. **According to the literature, the adherence to the treatment is limited, encouraging the need for supervised administration** (Kranzler & Soyka, 2018). It is not recommended as first-line treatment by National Institute for

Health and Care Excellence (NICE) (National Institute for Health and Care Excellence, 2011).

Another drug prescribed for the AUD treatment is **acamprosate** (EMA, 1996; FDA, 2004). Although its mechanism of action is not well known, it influences glutamatergic and GABAergic homeostasis, probably through N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors (Cano-Cebrián et al., 2003; Plosker, 2015). It is indicated to maintain abstinence rather than reduce alcohol drinking and is recommended as first-line treatment for AUD (National Institute for Health and Care Excellence, 2011), albeit **its efficacy remains controversial**. Some studies found that acamprosate showed positive effects in abstinence maintenance when compared with placebo, but other analysis could not confirm it (Plosker, 2015). Besides, some studies have shown that acamprosate is an inactive molecule whose effect on relapse prevention depends on its calcium component (Pradhan et al., 2018; Spanagel et al., 2014).

Another first-line treatment for AUD recommended by NICE is **naltrexone** (EMA, 1994; FDA, 1994). Naltrexone is a non-selective antagonist of opioid receptors (μ, κ and δ) that decreases the rewarding effects of alcohol consumption through the modulation of the dopaminergic mesolimbic pathway (Kranzler & Soyka, 2018). A systematic review conducted by Jonas and collaborators reported that no significant differences were found between acamprosate and naltrexone effects (Jonas et al., 2014). Besides, **side-effects** such as somnolence and gastrointestinal issues (nausea, abdominal pain, vomits) have been noticed after naltrexone administration (Kranzler & Soyka, 2018).

More recently, the opioid antagonist **nalmefene** was approved in Europe as an AUD treatment (EMA, 2013). This drug is a μ- and δ- opioid receptor antagonist and a partial agonist of κ-opioid receptor. It cannot be considered an anti-relapse drug since nalmefene was approved by the EMA for the "reduction of alcohol consumption in patients with alcohol dependence". Besides, **its approval was accompanied by controversy** because it is not clear if nalmefene provides advantages in comparison to the other approved treatments (Witkiewitz et al., 2019). Furthermore, nausea, dizziness, insomnia or headaches are some of the reported **side-effects** of this treatment (Kranzler & Soyka, 2018). Nalmefene is not approved by the FDA.

Finally, in 2018 the French health authorities (Agence nationale de sécurité du médicament et des produits de santé) approved the use of $GABA_B$ receptor agonist **baclofen** for AUD treatment, albeit its clinical use is extremely controversial because **the efficacy of baclofen treatment in AUD is questionable.** Some randomised clinical trials did not find benefits of baclofen treatment in comparison with placebo effects (Hauser et al., 2017; Reynaud et al., 2017). Moreover, further studies are needed to find the optimal dose since the relationship between baclofen dosage and the effect on AUD patients is not linear (Farokhnia et al., 2019). Some authors point out that the use of RS(±)-Baclofen as an AUD pharmacotherapy is premature (Minozzi et al., 2018) since the adverse effects of high doses of RS(±)-Baclofen should be explored in depth (Rolland et al., 2015) and more information about the dose-response relationship is needed (Agabio & Leggio, 2018). Consequently, preclinical studies are being performed to shed light on the adequacy of baclofen as AUD treatment. For example, a recent

preclinical study explored the efficacy of the treatment depending on the baclofen enantiomers (Echeverry-Alzate et al., 2021).

In summary, the current approved treatments show moderate rates of efficacy and low compliance rates magnified by its adverse side effects (Ch'Ng & Lawrence, 2018). That is why a plethora of research is focused on the discovery of **new therapeutic targets and the development of new pharmacotherapies for treating AUD** (Farokhnia et al., 2019; Franck & Jayaram-Lindström, 2013). Thus, in line with the literature, the present thesis tries to explore the behavioural and molecular effects of one potential candidate for promoting alcohol abstinence **NAC.**

2.4.2 NAC AND SUBSTANCE USE DISORDER

In 1963, the FDA approved NAC as a mucolytic agent for bronchopulmonary disorders. Since then, it has been marketed in oral, intravenous and nebuliser forms as an antidote for acetaminophen poisoning and mucolytic, but also as a dietary supplement (McClure et al., 2014). NAC is a cysteine prodrug commonly used as an **antioxidant** not only because it provides cysteine for GSH synthesis but also because it directly reduces disulphide bonds and acts as a direct scavenger of radicals (Aldini et al., 2018). It is a stable, safe, cheap, and well-tolerated molecule whose potential application as a treatment for different neurological disorders has piqued the interest of the scientific community (Bradlow et al. 2022; McClure et al. 2014; Smaga et al.,2021; Womersley et al. 2019).

PRECLINICAL AND CLINICAL EVIDENCE

There are several preclinical and clinical studies in the literature that have analysed the effects of NAC administration on SUDs. First and foremost, some studies regarding cocaine, heroin and nicotine misuse are briefly exposed:

Concerning preclinical studies, Amen and colleagues studied NAC effectiveness on **cocaine** misuse and found that NAC 60 mg/kg reduced cocaine-seeking behaviour in rats without altering its reinforcing properties (Amen et al., 2011). The effect of NAC on drug-seeking behaviour has also been tested for **heroin** in preclinical models. Thus, rats that had been trained for heroin self-administration received a 15-days treatment of NAC 100 mg/kg. The obtained results evidenced a reduction in heroin reinstatement and heroin-seeking behaviour in rats even 40 days after the last NAC injection (Zhou & Kalivas, 2008). Moreover, a dose-dependent effect was found since the increase of NAC dose (0, 30, 60 and 90 mg/kg) augmented the capability of attenuating heroin-seeking behaviour in rats (Hodebourg et al., 2019). Akin to these findings, the efficacy of different doses of NAC (30, 60 and 100 mg/kg) in reducing **nicotine**-seeking was evaluated in Wistar rats. It was found that a single dose of NAC 100 mg/kg could prevent nicotine-seeking behaviour during reinstatement. This effect was specific since the same NAC dose did not alter saccharin-seeking behaviour (Moro et al., 2018).

In the clinical setting, meta-analyses of NAC clinical trials support the use of NAC in ameliorating drug craving, although in some trials NAC failed to reduce it (Deepmala et al., 2015; Duailibi et al., 2017). To

provide concrete evidence, some authors found that NAC 1200 or 2400 mg/day could lessen cocaine craving, although it did not alter the euphoric properties (Amen et al., 2011). In the same line, Woodcock and colleagues recently found that in some situations 3600 daily mg of NAC is able to reduce **cocaine**-seeking behaviour in human patients (Woodcock et al., 2021).

Regarding **ethanol** consumption, existing preclinical research has already demonstrated some advantageous effects over ethanol consumption, ethanol-seeking and ethanol relapse. Firstly, Quintanilla and collaborators demonstrated that NAC (30 mg/kg or 60 mg/kg) can reduce voluntary chronic ethanol consumption during the maintenance phase but not during the initial steps of ethanol intake, the so-called acquisition phase (Quintanilla et al., 2016). Later, this group also reported that oral NAC administration for 14 days reduced ethanol binge-like drinking behaviour, determined after an abstinence period, but only 60 minutes of ethanol re-access were assessed (Quintanilla et al., 2018). These preclinical experiments conducted by Quintanilla and colleagues were performed with female UchB rats, **a line of rats bred and selected for their high rate of ethanol consumption** (Quintanilla et al., 2006). Furthermore, the group headed by Dr. Naassila has reported additional data. Concretely, in a model of binge-drinking in which rats could self-administer 20% of ethanol for 15 minutes, NAC 100 mg/kg demonstrated a reduction in ethanol consumption, motivation, seeking and relapse (Lebourgeois et al., 2018). This group also reported that NAC 50 mg/kg did reduce ethanol reinstatement after one month of abstinence in dependent animals (Lebourgeois et al., 2019). Curiously,

in these experiments, **only a single dose of the drug** was intraperitoneally injected 60 min before the experimental session.

In clinical studies, some promising results were reported. Hence, when human AUD patients with a posttraumatic stress disorder were treated with NAC 2400 mg/day for 8 weeks, a reduction in ethanol craving was found (Back et al., 2016). Moreover, AUD patients receiving NAC 2400 mg/day for 12 weeks also reported fewer weekly drinks (Squeglia et al., 2018). Nonetheless, according to the literature, further research is necessary to confirm NAC effectiveness as a treatment for AUD patients (Smaga et al., 2021).

Additionally, several papers have recently reported sex differences in the psycho-pharmacological effects of NAC under different indications and experimental paradigms such as schizophrenia (Monte et al., 2020) or SUD's treatment (Goenaga et al., 2020). In the case of SUDs, preclinical research determined that the NAC 100 mg/kg effect on nicotine-seeking behaviour worked only in male, but not in female rats (Goenaga et al., 2020). However, positive effects were previously reported after the administration of NAC 100 mg/kg in female UChB rats. Specifically, NAC was able not only to block nicotine-reinstatement in a model of conditioned place preference (CPP) but also reduced voluntary nicotine oral consumption (Quintanilla et al., 2018).

Potential Mechanisms of action

In addition to the afore-mentioned antioxidant effects of NAC, **glutamatergic properties** have been attributed to this drug. Thus, Dr. Kalivas and colleagues have proposed a mechanism of action that could explain both properties: as depicted in figure 7, in the pro-oxidant

conditions of the brain after drug consumption, NAC is deacetylated into cysteine which dimerises to form cystine. The increased extracellular cystine levels would stimulate the activity of system xC- that introduces one molecule of extracellular cystine in the astrocytes and expels one molecule of glutamate to the extracellular space. Consequently, on one hand, cystine could be reduced to cysteine in the astrocyte, acting as substrate for GSH synthesis. On the other hand, elevated extrasynaptic glutamate levels would stimulate presynaptic mGluR2/3 receptor, thereby reducing the release of glutamate in the synaptic cleft. Thus, according to this hypothesis, the administration of NAC may have a direct impact on oxidative stress status and the normalisation of the glutamatergic transmission (Kalivas, 2009; Womersley et al., 2019). Other authors add that the pro-oxidant conditions may impair GLT1 activity. The increase in GSH due to NAC administration would restore its activity (Berríos-Cárcamo et al., 2020).

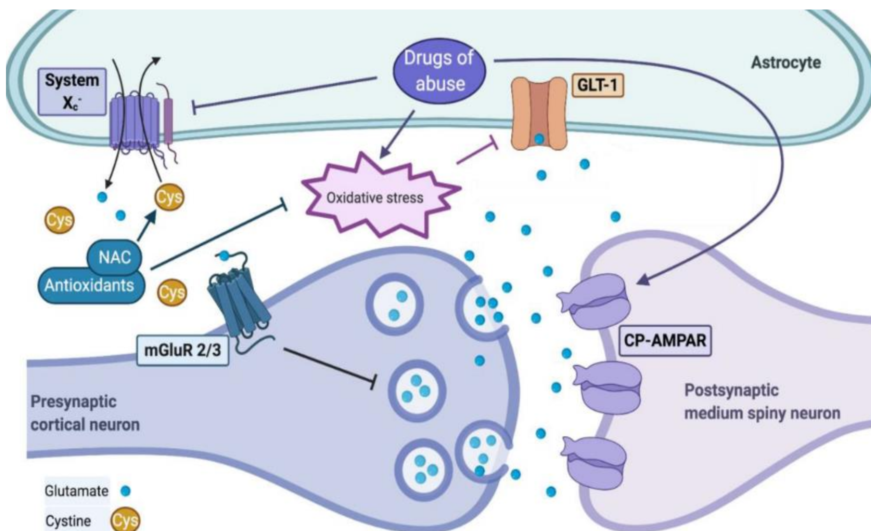


Figure 7. Potential effect of NAC administration on the glutamatergic synapse in the presence of drugs of abuse. Blue dots represent glutamate. Drugs of abuse increase oxidative stress which impairs GLT1 activity. NAC supplies cystine (Cys) which is introduced in the astrocyte by system xC- in exchange with glutamate causing an augmentation in the extracellular glutamate levels. This scenario activates mGluR2/3 that inhibits glutamatergic synapse. Besides, the presence of cystine increases GSH production reducing oxidative stress, which also recovers GLT1 activity. Adapted from (Berríos-Cárcamo et al., 2020).

Nonetheless, another aspect should be considered. As previously mentioned in section 2.2.1, glutamate has higher affinity for mGluR2/3 than for mGluR5, but high glutamate levels can also activate mGluR5. Thus, an *in vitro* experiment demonstrated that NAC, depending on the administered dose, has inhibitory and excitatory effects on the glutamatergic transmission onto nucleus accumbens. Concretely, NAC augments the extracellular glutamate concentrations and, this event inhibits the glutamatergic synapse by activating mGluR2/3 (low NAC dose) or excites the postsynaptic neuron by activating the mGluR5 (high NAC dose) (Kupchik et al., 2012).

Regarding NAC glutamatergic properties, other molecular mechanisms have also been explored in order to explain the positive effects associated with NAC administration for the treatment of several SUDs. In particular, several studies have investigated if NAC is able to regulate the expression of different proteins involved in glutamate homeostasis such as GLT1, or xCT. As previously commented, literature has evidenced the existence of significant alterations in the expression of these proteins in nucleus accumbens after ethanol exposure (summarised in Table 1). Next, in Table 2 reported evidence about the effects of NAC administration on the expression of GLT1 and xCT

proteins in nucleus accumbens after cocaine, nicotine and ethanol exposure is summarised.

Experimental condition	NAC dose	Rats	GLT1	xCT	Reference
Cocaine-seeking	100 mg/kg i.p.	♂ SD	⇧	⇧	Knackstedt , 2010
Cocaine-seeking	100 mg/kg i.p.	♂ SD	⇧		Reissner, 2015
Reinstatement cocaine SA	60 mg/kg i.p.	♂ SD	⇧		Ducret, 2015
Nicotine-seeking	100 mg/kg i.p.	♂ Wistar	⇧	= ⇧ *	Moro, 2020
Nicotine.seeking	100 mg/kg i.p.	♂ SD	⇧		Namba, 2020
Ethanol exposure	120 mg/kg i.p.	♂ Swiss mice		=	Morais-Silva, 2016
8 hours ethanol abstinence	100 mg/kg i.p.	♂ Wistar	⇩	=	Lebourgeois, 2019
Naïve	120 mg/kg i.p.	♂ Swiss mice		=	Morais-Silva, 2016
Naïve	100 mg/kg i.p.	♂ SD	=		Namba, 2020
Naïve	100 mg/kg i.p.	♂ Wistar	=	=	Moro, 2020

Table 2. NAC effects on GLT1 and xCT protein expression in nucleus accumbens under different experimental conditions: ***cocaine****-seeking, reinstatement of cocaine self-administration (SA),* ***nicotine****-seeking,* ***ethanol*** *exposure or early abstinence and no drug exposure (naïve). * indicates that two experiments were performed and the obtained results were different: an experiment performed after 7 days of nicotine-seeking test did not find differences in xCT expression after NAC administration; when performed on day 51, NAC augmented xCT expression. If the research did not report some data, the box is empty. SD=Sprague-Dawley rat strain.*

As can be seen in Table 2, some authors reported that NAC is able to upregulate the expression of GLT1 after reinstatement of cocaine self-

administration or during the experience of cocaine and nicotine-seeking behaviour. However, studies involving NAC and ethanol exposure are scarce. Apparently, collected data related to xCT suggest that the presence of NAC during cocaine and nicotine-seeking experiments increases xCT expression in nucleus accumbens. However, according to the little data reported, NAC does not affect the expression of xCT in drug naïve conditions or after ethanol exposure or abstinence. Furthermore, no evidence about how NAC affects GLAST expression was found in literature neither under drug naïve nor under drug exposure conditions. Hence, it may be argued that few studies have explored the effects of NAC on the expression of GLT1 and xCT in the nucleus accumbens.

In addition, NAC has been proven to perform **anti-inflammatory** effects under *in vitro* or *in vivo* conditions. For instance, it reduces TNF-α-induced NfκB activation in cell culture (Oka et al., 2000) as well as plasma inflammatory markers in diabetic rats (Tsai et al., 2009). These effects, together with the afore-mentioned role as an antioxidant, pointed to NAC as a potential pharmacotherapy for several SUDs. In fact, several preclinical studies that explore the effect of NAC in AUD are accompanied by evidence about its antioxidant and anti-inflammatory effects in rats (Israel et al., 2021; Quintanilla et al., 2018, 2020; Schneider et al., 2017) or in zebrafish (Mocelin et al., 2018).

Lastly, given that NAC is a pro-drug of cysteine and cystine, another mechanism may potentially be involved in its effects: in the last decades, consistent results have demonstrated the involvement of brain-derived ACD and/or its bio derivatives (such as salsolinol), in the ethanol actions in the mesocorticolimbic pathway. Hence, numerous

authors claim that motivational and neuropharmacological effects of ethanol are mediated, at least in part, by them (Deehan et al., 2013; Peana et al., 2016). **ACD is a highly reactive molecule, therefore, it is capable of being "sequestered" by thiol amino acids** such as D-penicillamine or L-cysteine, which react non-enzymatically with ACD to form stable non- toxic adducts (Kera et al., 1985; Nagasawa et al., 1980). Consequently, these thiol amino acids have been able to reverse some of the neurobiological effects of ethanol (Martí-Prats et al., 2010, 2015a; Orrico et al., 2013; Peana et al., 2009; Sirca et al., 2011). Therefore, the potential interaction between L-cysteine and brain-derived ACD after ethanol consumption could also be involved in NAC anti-relapse effects. Figure 8 includes the chemical structure of both molecules NAC and L-cysteine.

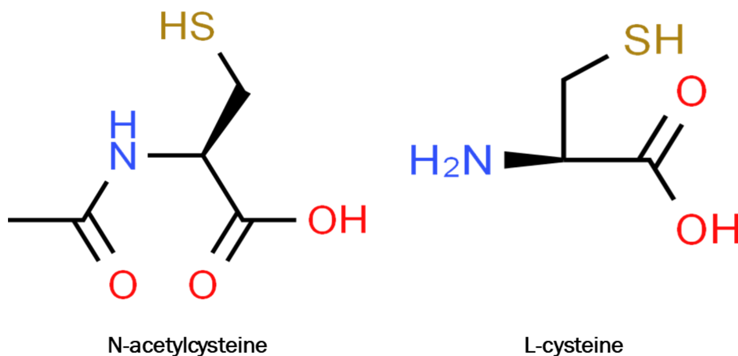


Figure 8. Chemical structure of NAC on the left and L-cysteine on the right.

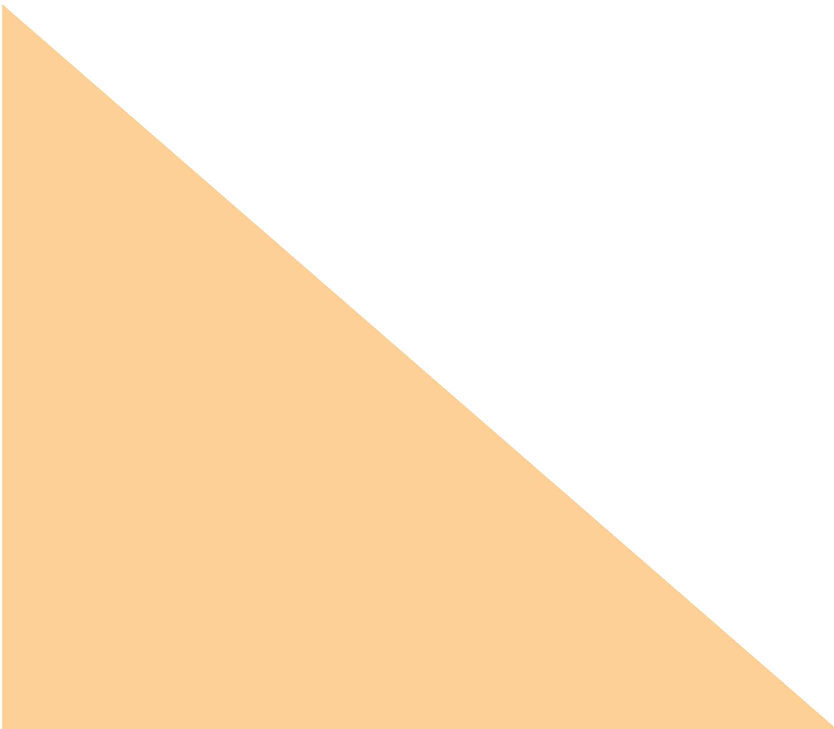
FUTURE DIRECTIONS

Despite the scientific efforts addressed to study the effectiveness of NAC as an AUD treatment, until now **there is no evidence about its**

ethanol anti-relapse effect using (i) a robust preclinical model, such as the ADE model and (ii) a wild-type rat strain. In our opinion, testing NAC under the **ADE preclinical model with non-preferent animals** would provide additional information not only about its anti-relapse effect but also about the underlying mechanisms of action. This idea is supported by a recent review that gathers preclinical and clinical evidence of NAC as a treatment for SUDs. This paper points out that although NAC has demonstrated, in some cases, an ability to reduce craving, consumption, drug-seeking, relapse or abstinence symptoms to different drugs of abuse in animal models, **the mechanism of action of NAC is not completely understood**. Thus, the authors propose to perform further studies to explore its effects on glutamatergic homeostasis, neuroinflammation and oxidative stress in order to unravel the molecular mechanism underlying NAC effects on SUD. Likewise, more clinical research is needed to assess the efficacy of NAC as a treatment for SUD patients (Smaga et al., 2021).

Finally, some preclinical studies with NAC have previously noted sex-related differences (Goenaga et al., 2020; Monte et al., 2020). Moreover, the National Institute of Health of the United States stands up for the importance of biochemical research with **gender perspective**. In the same line, the National Institute on Drug Abuse (NIDA) in the United States is focused on studying substance use disorders considering sex differences (Gipson et al., 2021). Accordingly, the experimental design of the present thesis is going to be focused on all these gender aspects.

3. OBJECTIVES



Generally, the present thesis studies, from a biochemical point of view, the dynamics of alcohol withdrawal and relapse. The main objective is to explore the potential pathways that could be involved, at least in part, in triggering the relapse behaviour, being able to be proposed as targets to address new anti-relapse pharmacotherapies for AUD. Furthermore, this project proposes and studies NAC as a plausible treatment for preventing alcohol relapse. Hence, to accomplish these aims, five specific objectives were tracked:

01. Characterise the relapse-like drinking behaviour in non-selected cohorts of male and female Wistar rats that have long-term been exposed to alcohol consumption under the ADE preclinical model. Obtained results may allow us to identify the most vulnerable subpopulations.

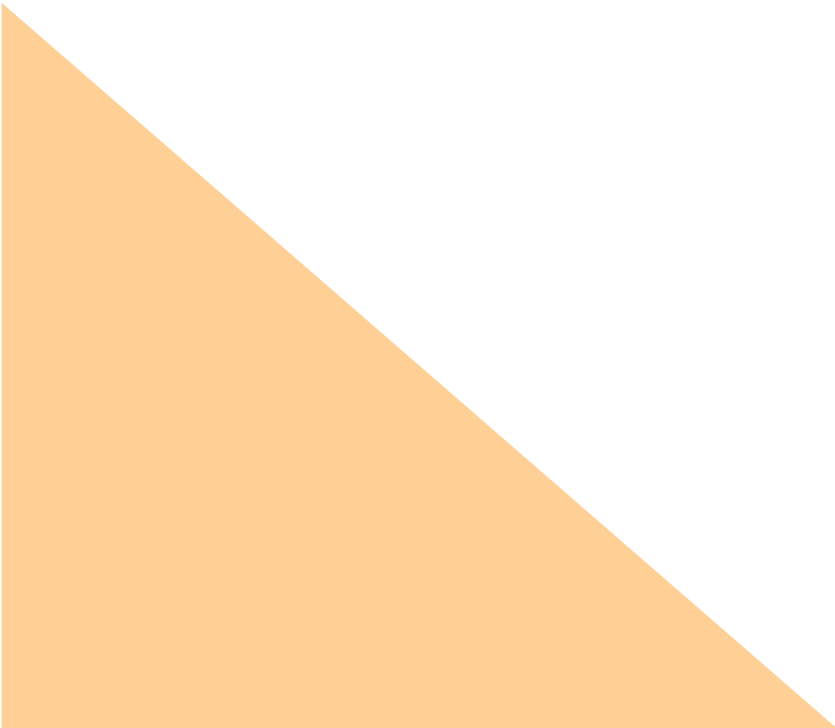
02. Evaluate the effect of NAC in the prevention of alcohol relapse by exploring its ability to blunt the ADE manifestation in vulnerable subpopulation of male and female Wistar rats after long-term alcohol exposure.

03. To explore the existence of correlations between the male rats' vulnerability to display alcohol relapse-like drinking behaviour and the existence of different biochemical alterations in the brain during the abstinence period. The influence of alcohol reintroduction will also be explored. Concretely, brain oxidative status and several neuroinflammatory mediators will be analysed.

04. Analyse the potential mechanism by which NAC is able to prevent the alcohol relapse in male rats. Particularly, the effects that NAC administration may have on oxidative stress, neuroinflammation and glutamatergic homeostasis are going to be analysed.

05. Finally, a preliminary exploration of the effects of NAC after acute administration in the activation of MCLS and the underlying mechanisms will also be performed.

4. METHODS



4.1. Study of the neurobiology of abstinence and relapse to alcohol consumption. Evaluation of NAC as a potential anti-relapse pharmacotherapy

For the study of this first section of the present thesis, the following experiments were carried out:

- Experiment 1. Identification and characterisation of two subpopulations of animals depending on their alcohol relapse-like drinking behaviour.

- Experiment 2. Study of the efficacy of NAC in the prevention of alcohol relapse-like drinking behaviour.

- Experiment 3. Brain oxidative and neuroinflammatory status determined in male Wistar rats after 21 days of ethanol abstinence. Effects of alcohol reintroduction.

- Experiment 4. Unravelling the mechanism of action underlying NAC anti-relapse effect.

The experimental procedures and the design of each experiment is detailed in the following epigraphs.

4.1.1 Animals

All the animals were purchased from Envigo (Barcelona, Spain). Behavioural experiments using Wistar adult rats were performed in the Central Service for Experimental Research Support (SCSIE) of the University of Valencia. Specifically, in the animal facilities of the Faculty of Pharmacy. Animals were housed in plastic cages (48 x 38 x 21 cm^3) under controlled humidity (60%), controlled temperature (22 ºC) and 12 h light–dark cycle. All animals always had free access to standard solid diet and water.

58 male Wistar rats (weighing 356 ± 3.2 g at the beginning of the experiment) and 38 female Wistar rats (weighing 225 ± 1.7 g at the beginning of the experiment) were purchased. All these animals were individually housed as previously explained.

All the procedures were performed in strict accordance with EEC Council Directive 2010/63/EU, Spanish laws (RD 53/2013) and animal protection policies. The Animal Care Committee of University of Valencia and the regional government (Conselleria de Agricultura, Medio Ambiente y Cambio Climático) approved and authorised all experiments. The authorised procedures references were: 2016/VSC/PEA/00180, 2017/VSC/PEA/00099 and 2019/VSC/OEA/0282.

4.1.2 DRUGS

Ethanol was purchased from Scharlau S.A (Madrid, Spain) as 96% v/v ethanol. For alcohol consumption protocols, ethanol was diluted with tap water to a final concentration of 5%, 10% or 20% v/v.

NAC ($C_5H_9NO_3S$) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Its molecular weight is 163.19 g/mol and its chemical structure is depicted in Figure 9.

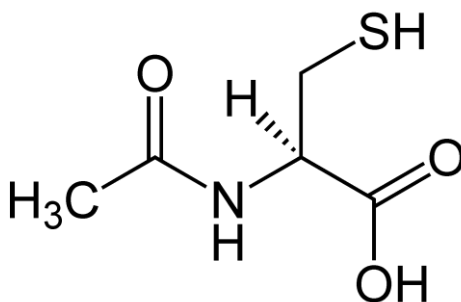


Figure 9. Chemical structure of NAC.

When administered through subcutaneous injection, it was freshly diluted in phosphate buffer (PB) 0.4 M to a final concentration of 15, 60 or 100 mg/ml. Then pH was adjusted to 7.4 with NaOH.

4.1.3 EXPERIMENTAL PROCEDURE

4.1.3.1 LONG-TERM VOLUNTARY ALCOHOL DRINKING WITH REPEATED DEPRIVATION PHASES (ADE MODEL)

We considered that the most suitable preclinical model to achieve objective 1 and study the relapse-like drinking behaviour in rats was the experimental procedure based on **the manifestation of the ADE.** As previously explained, this model consists of a long-term alcohol exposure protocol that combines random periods of alcohol access with random periods of alcohol deprivation. According to the literature, the manifestation of the ADE phenomenon after a deprivation stage mimics the relapse-like drinking behaviour observed in humans (Leong et al., 2018).

During ethanol availability periods (referred as "consumption periods"), animals had free access to food and four bottles that contained tap water and three different dilutions of ethanol (5, 10 and 20% v/v). On the contrary, throughout abstinence phases (also referred as "deprivation periods"), ethanol bottles were withdrawn. Hence, animals only had access to food and water *ad libitum*.

For the execution of this research project, two different cohorts of rats were used: one with 43 male Wistar rats and another one with 38 female Wistar rats. The duration of each period is detailed in Table 3 and Table 4, respectively.

Stage	Experimental procedure	Duration (weeks)	Total weeks
	HABITUATION	2	2
1	CONSUMPTION PERIOD	9	11
	ABSTINENCE PERIOD	2.5	13.5
2	CONSUMPTION PERIOD	5.5	19
	ABSTINENCE PERIOD	2.5	21.5
3	CONSUMPTION PERIOD	7.5	29
	ABSTINENCE PERIOD	1.5	30.5
4	CONSUMPTION PERIOD	3.5	34
	ABSTINENCE PERIOD	3	37
5	CONSUMPTION PERIOD	1.5	38.5
	ABSTINENCE PERIOD	3	41.5

Table 3. Duration of each experimental period and the accumulated duration of the entire procedure (expressed in weeks), when the ADE protocol was performed with a cohort of ***male Wistar rats*** *(n=43)*

Stage	Experimental procedure	Duration (weeks)	Total weeks
	HABITUATION	2	2
1	CONSUMPTION PERIOD	8	10
	ABSTINENCE PERIOD	2.5	12.5
2	CONSUMPTION PERIOD	3.5	16
	ABSTINENCE PERIOD	1.5	17,5
3	CONSUMPTION PERIOD	4	21,5
	ABSTINENCE PERIOD	3,5	25
4	CONSUMPTION PERIOD	4	29
	ABSTINENCE PERIOD	3.5	32.5
5	CONSUMPTION PERIOD	3	35,5

Table 4. Duration of each experimental period and the accumulated duration of the entire procedure (expressed in weeks) when the ADE protocol was performed with a cohort of ***female Wistar rats*** *(n=38)*

The total duration of the ADE protocol performed with male Wistar rats was around 10 months, while female Wistar rats underwent the ADE protocol during 9 months.

As can be observed in Table 3 and Table 4, before animals were exposed to ethanol consumption, rats experienced two weeks of habituation to individual housing. Immediately after, ethanol bottles were supplied to rats. Since then, animals were exposed to five random alcohol exposure and alcohol deprivation periods. According to the literature, the duration of each alcohol exposure and deprivation phases was deliberately different to avoid behavioural adaptations (Vengeliene et al., 2007).

As a normal routine, during alcohol exposure periods, the alcohol intake was determined twice a week. In addition, to measure ADE, alcohol consumption was determined daily during the three days before and after each abstinence period. To do so, bottles were removed from cages and weighed. In order to avoid the development of place preferences, the position of bottles in the cages was changed every measure day (Vengeliene et al. 2005).

The registered data was used to calculate different parameters:

Ethanol intake was expressed as g/kg/day for each animal. The consumed volume of each experimental solution (ethanol 5%, 10% or 20% v/v) during a period was calculated by weighing each bottle before and after the considered period **Δv**. Ethanol density (0.81 g/ml) and the concentration of ethanol were considered to determine the ethanol intake from each bottle. The obtained result was normalised with respect to the weight of the animal (expressed in kg) and the number of days elapsed. Equation 1 summarises the performed calculation.

$$Ethanol\ intake = \frac{0.81 * (5 * \Delta\text{v ethanol } 5\% + 10 * \Delta\text{v ethanol } 10\% + \ 20 * \Delta\text{v ethanol } 20\%)}{weight\ (kg) * number\ of\ days * 100}$$

Equation 1. Ethanol intake calculation (g/kg/day). Δv.represents the consumed volume of each bottle and 0.81 is the ethanol density. Total ethanol consumed volume and density were used to calculate ethanol intake (g). The result was normalised by the number of days elapsed and the rat weight (kg).

Total fluid intake (ml/day) was calculated as the addition of the recorded Δv from each bottle (water, ethanol 5% v/v, ethanol 10% v/v and ethanol 20% v/v) normalised by the number of days considered. The calculation is illustrated in Equation 2.

$$Total\ fluid\ intake = \frac{\Delta\text{v water} + \Delta\text{v ethanol } 5\% + \Delta\text{v ethanol } 10\% + \ \Delta\text{v ethanol } 20\%}{number\ of\ days}$$

Equation 2. Calculation of total fluid intake (ml/day). The consumed volume (Δv) from each bottle was divided with respect to the number of days of the considered period.

Total ethanol preference (%) was calculated as the addition of the recorded Δv from each ethanol bottle (ethanol 5%, 10% or 20% v/v) multiplied by 100 and divided by total fluid intake. Equation 3 represents this calculation.

$$Total\ ethanol\ preference =$$

$$100 * \frac{\Delta\text{v ethanol 5\%} + \Delta\text{v ethanol 10\%} + \Delta\text{v ethanol 20\%}}{\text{Total fluid intake}}$$

Equation 3. Total ethanol preference (%). The consumed volume (Δv) of each ethanol solution was added and divided by total fluid intake.

Particular (X%) ethanol preference (%) was calculated as the Δv from a concrete ethanol bottle multiplied by 100 and divided by the total volume of ethanol consumed (Δv ethanol 5% v/v, Δv ethanol 10% v/v and Δv ethanol 20% v/v)

$$Ethanol\ X\%\ preference =$$

$$100 * \frac{\Delta\text{v ethanol X\%}}{\Delta\text{v ethanol 5\%} + \Delta\text{v ethanol 10\%} + \Delta\text{v ethanol 20\%}}$$

Equation 4. Ethanol preference of (X) concentration (%). The consumed volume (Δv) of one ethanol solution was divided by total ethanol consumed volumes.

The calculation of ethanol intake, total fluid intake, total ethanol preference, and particular (5%, 10% or 20 %v/v) ethanol preference was used in the performance of experiment 1 and experiment 2.

4.1.3.2 Euthanasia and brain dissection

To euthanise animals, they were deeply anaesthetised with isoflurane (Esteve veterinaria, Barcelona, Spain) before cervical dislocation. Then, brains were removed and used as described below.

Biochemical techniques were used to analyse different parameters in specific brain areas. After removal, brains were directly frozen at -80ºC and kept at this temperature until dissection. By using a scalpel and a slide gauge, specific brain coronal sections were obtained. The procedure was based on the coordinates described in the Rat brain atlas (Paxinos & Watson, 2007).

Figure 10 shows brain coronal images including the selected brain areas. A portion from Bregma 4.2 mm until Bregma 2.76 mm was dissected to extract **PFC** (Figure 10 A). The portion from Bregma 2.52 mm to Bregma 0.96 mm was used to dissect the **nucleus accumbens** and **DS** (Figure 10 B). Finally, a section from Bregma -2.52 mm to Bregma -3.36 mm was used to extract the **HIP** and **AMG** (Figure 10 C).

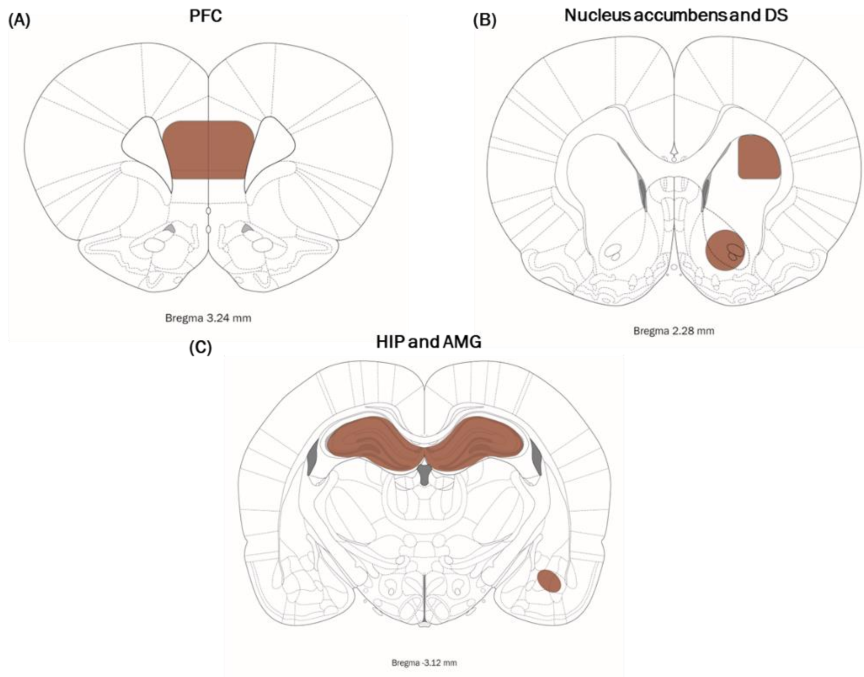


Figure 10. Representative images of coronal slices of the rat brain from Paxinos and Watson (2007). Bregma coordinate is specified under each brain slice. (A) PFC is coloured in brown. (B) DS is coloured in brown at the top. Nucleus accumbens portion is signalled by a brown circle. (C) On the top, HIP is coloured and AMG is pointed at the bottom. Adapted from Paxinos & Watson, 2007

As subsequently detailed, the PFC was used to determine the expression of neuroinflammatory mediators whereas nucleus accumbens and DS were used in the analysis of the expression of different proteins associated with glutamate homeostasis (GLT1, GLAST and xCT). Finally, HIP and AMG were used to measure the oxidative status.

4.1.3.3 Ultraperformance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS)

In order to examine the oxidative stress levels in the HIP and AMG, the ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used. This technique allowed us to measure levels of GSH and GSSG (experiments 3 and 4).

Working solutions

— **Phosphate buffer saline (PBS)** 10X was prepared and stored at room temperature: 3.9 g of disodium phosphate dihydrate, 10.65 g of disodium phosphate and 83 g of sodium chloride were dissolved in distilled water until 1 litre. PBS 1X was prepared before each use mixing 100 ml of PBS 10X and 900 ml of distilled water.

Sample preparation

Frozen HIP and AMG tissues were homogenised by using a dispersing tool (Ultra-Turrax T25, Labortechnik) in PBS and 10 mmol/L N-ethylmaleimide (NEM) purchased from Sigma-Aldrich (St. Louis, MO, USA) (pH 7.0). A ratio tissue:buffer of 1:4 was always used. Then, perchloric acid solution was added until a final concentration of 4% was obtained. Samples were centrifuged at 11000 rpm for 15 min at 4 °C. 20 µl of each supernatant were injected in the chromatographic system (UPLC-MS/MS) located in the SCSIE of the University of Valencia. The pellet of each sample was used for the quantification of protein concentration. For this purpose, the pellet was resuspended in NaOH

1N in the same volume of the collected supernatant (see section 4.1.3.6 for protein quantification protocol).

MASS SPECTROMETRY

The chromatographic system consisted of a Micromass QuatroTM triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Zspray electrospray ionisation source operating in the positive ion mode with a LC-10A Shimadzu (Shimadzu, Kyoto, Japan) coupled to the MassLynx software 4.1 for data acquisition and processing. Samples were analysed by reversed phase UPLC with a C18 Mediterranea SEA column (Teknokroma, Barcelona, Spain) with 5µm particle size (0.60x21 cm). The mobile phase consisted of the following gradient system (min/%A/%B) (A, 0.5% formic acid; B, isopropanol/acetonitrile 50/50; 0.5% formic acid): 5/100/0, 10/0/100, 15/0/100, 15.10/100/0, and 60/100/0. The flow rate was set at 0.2 ml/min. Positive ion electrospray tandem mass spectra were recorded with the electrospray capillary set at 3 kV and a source block temperature of 120 °C. Nitrogen was used at flow rate of 500 L/h for drying and at 30 L/h for nebulizing. Argon at 1.5610^{-3} mbar was used as the collision gas for collision-induced dissociation. An assay based on UPLC-MS/MS with multiple reaction monitoring was developed using the transitions *m/z*, cone energy (V), collision energy (eV) and retention time (min) for each compound that represents favourable fragmentation pathways for these protonated molecules (Table 5).

Analyte	Cone (V)	Collision (eV)	Transition (m/Z)	Retention time (min)
GSH	30	15	433>304	4,32
GSSG	30	25	613>355	1,46

Table 5. Transitions and retention times for the determination of GSH and GSSG by UPLC–MS/MS.

Calibration curves were obtained using twelve-point (0.01–100 mmol/l) standards (Sigma-Aldrich, St Louis, MO, USA) for each compound. The concentrations of analytes were expressed as nmol/mg of protein.

4.1.3.4 Reverse Transcription - quantitative PCR

In order to analyse the gene expression levels of several neuroinflammatory modulators, the Reverse Transcription – quantitative PCR (RT-qPCR) was the selected technique. Concretely, mRNA levels of IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3 in the PFC of rats were determined (experiments 3 and 4).

RNA extraction

RNA was extracted from the PFC using Trizol reagent (Sigma-Aldrich, St Louis, MO, USA). For that purpose, each sample tissue was mechanically disaggregated in 1 ml of trizol using a dispersing tool (Ultra-Turrax T25, Labortechnik). Then, samples rested for 5 minutes in ice, and 200 µl of chloroform was added. The mixture was centrifuged at 12000g during 15 minutes at 4ºC to separate three phases: a lower organic pink phase that contained proteins, a white interface with DNA, and an upper aqueous phase with RNA. The supernatant that contained RNA was mixed with 500 µl of isopropanol and rested in ice for 10 minutes before being centrifuged at 12000g during 10 minutes at 4ºC. After that, the RNA was present in the pellet. The supernatant was removed and the pellet was resuspended in 1 ml of ethanol 75%. Samples were centrifuged at 7500g during 5 minutes at 4ºC and then the supernatant was removed. The pellet dried on ice for 15 minutes to let ethanol evaporate. Finally, it was dissolved in Nuclease-Free water (Ambion, Thermo Fisher Scientific, Rockford, IL, USA) and incubated for 10 minutes at 60ºC in a block heater. The purity and concentration of

RNA was measured in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Reverse transcription

First-strand cDNA synthesis was performed with the NZY First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) according to the manufacturer's instructions: for each sample, 15 µl of RNA (approximately 1000 ng) were mixed with 1 µl of random hexamer mix (50 ng/ µl), 1 µl of dNTP mix (0.5 mM), 2 µl of 10X reaction buffer, 1 µl of ribonuclease inhibitor (40 U/ µl) and 1 µl of reverse transcriptase (20 U/ µl). All the reactives were provided by a commercial pack. Reverse transcription was performed in the thermocycler Mastercycler® ep (Eppendorf, Hamburg, Germany) in three steps: 10 minutes at 25ºC, 40 minutes at 50ºC and 5 minutes at 98ºC. After that, 1 µl of RNAsa H was added and samples were incubated for 20 minutes at 37ºC to eliminate RNA residues. Samples were stored at -20ºC until use.

Conventional PCR

Different oligonucleotide sequences were designed for cDNA amplification (Table 6). They were purchased from Integrated DNA Technologies (Coralville, IA, USA). In order to check the efficiency and the proper amplification temperature of each pair of primers, conventional PCR were performed. Each mix contained: 1 µl of cDNA sample, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of Nuclease-Free water (Ambion, Thermo Fisher Scientific, Rockford, IL, USA) and 5 µl of PCR Master Mix (Ambion, Thermo Fisher Scientific, Rockford, IL, USA).

Steps for PCR were: 5 minutes at 94ºC and 40 cycles (20 seconds at 94ºC, 20 seconds at 60ºC and 20 seconds at 72ºC) and a final step of 5 minutes at 72ºC.

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Gene	Forward Primer 3' – 5'	Reverse primer 5'-3'
PPIA	TGTGCCAGGGTGGTGACTTT	CGTTTGTGTTTGGTCCAGCAT
IL-1β	CAGCAGCATCTCGACAAGAG	CATCATCCCACGAGTCACAG
IL-6	TGTGCAATGGCAATTCTGAT	CGGAACTCCAGAAGACCAGAG
TNF-α	GGTGGGCTGGGTAACAAGTA	TTCAAGATGGTGCCAAAGTG
HMGB1	ATCTAAATACGGATTGCTCAGGAA	AGGGACAAACCACAATATAGGAAAA
iNOS	ACCAGCACCTACCAGCTCAA	CCCTTTGTTGGTGGCATACT
NfκB	CAAGAGTGACGACAGGGAGAT	GCCAGCAGCATCTTCACAT
NLRP3	CCCTCATGTTGCCTGTTCTT	TCCAGTTCAGTGAGGCTCTG

Table 6. Primer sequences used. Cyclophilin A (PPIA) was used as a housekeeping gene in the experiment. Then the studied genes were: IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3.

Amplicons were electrophoretically detected in 2% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Landing Parkway Fremont, CA, USA).

cDNA AMPLIFICATION

Once mRNA was reverse transcribed into cDNA, its expression level was analysed by Real Time-PCR (RT-PCR). For that purpose, 1 µl of cDNA was mixed with 0,25 µl of forward primer, 0.5 µl of reverse primer, 1.5 µl of Nuclease-free water and 2 µl of LightCycler FastStart Universal SYBR Green I Master (Roche Diagnostics, Basel, Switzerland). Each sample was analysed in triplicate.

RT-PCR was performed in a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland). The amplification conditions were:

10 minutes at 95°C and 40 cycles (20 seconds at 95°C, 20 seconds at 63°C and 20 seconds at 72°C). After that, samples remained at 40°C. Data was analysed with LightCycler 480 SW 1.5 software (Roche Diagnostics, Basel, Switzerland). Melting curves were checked to ensure that only a single product was amplified and the threshold cycle (CT) was determined. Triplicates with difference greater than 0.5 cycles were discarded. The relative gene expression was expressed as follows: fold change= $2^{-\Delta(\Delta CT)}$, where ΔCT = CT target – CT housekeeping, and Δ(ΔCT) = ΔCT treated - ΔCT control. Cyclophilin A (PPIA) was used as a housekeeping gene to normalise the transcription analysis.

4.1.3.5 Western Blot

The western blot technique was used in experiment 4 to relatively quantify the levels of proteins involved in glutamate homeostasis: GLT1, xCT and GLAST. Two areas of the striatum were analysed: the DS and the nucleus accumbens.

Working solutions

For performing the western blot assays the following solutions were used:

- For protein extraction a radio-immunoprecipitation assay (**RIPA**) **lysis buffer** was used. It was freshly prepared in PBS 1X before each use. The RIPA lysis buffer contained: 3.5 mM sodium dodecyl sulphate, 12 mM sodium deoxycholate, 1% (v/v) Igepal CA-630 and 1% protease inhibitor cocktail from Roche Diagnostics (Basel, Switzerland).
- A **Loading buffer** was the vehicle to load protein samples into the electrophoresis gel. It was filtered through a 0.2 µm pore filter and stored at -20 °C until use. 10 ml of loading buffer 4x solution contains: 5 ml of Tris 0.5 M, 4 ml of glycerol, 0.8 g of SDS, 1 ml of β-mercaptoethanol and 4 mg of bromophenol blue.
- A **Running buffer** was used to carry out electrophoresis. One litre of 10X running buffer was prepared as follows: 144.12 g of glycine, 30.28 g of trizma base and 10 g of sodium dodecyl sulphate in distilled water. It was used at 1X concentration by

diluting 100 ml of the running buffer 10X with 900 ml of distilled water.

— A **Transfer buffer** was used for protein transference from the electrophoresis gel to a nitrocellulose membrane. One litre of 10X transfer buffer includes: 30.25 g of trizma base and 144.2 g of glycine in distilled water. For use, 100 ml of transfer buffer 10X was mixed with 200 ml of methanol and 700 ml of distilled water.

— **Trizma buffer saline (TBS)** was the solution used for washing membranes. It was prepared in a 10X concentration: 24.4 g of trizma base and 80 g of sodium chloride in 1 litre of distilled water (being the pH adjusted to 7.6). It was normally used with Tween 20 0.1% to avoid contaminations (TBS-T 0.1%): 100 ml of TBS 10X, 1 ml of Tween 20 and 899 ml of distilled water.

— A **blocking solution** was useful for blocking nonspecific bindings of antibodies. Its composition was 5% non-fat dried milk in TBS-T 0.1%.

PROTEIN EXTRACTION AND QUANTIFICATION

Frozen samples from DS and nucleus accumbens were mechanically homogenised with a dispersing tool (Ultra-Turrax T25, Labortechnik) in RIPA lysis buffer on ice. Homogenates were kept on ice for 30 minutes and centrifuged at 14000 rpm for 15 minutes. The supernatant was collected, and protein content was quantified using the Bradford Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

For protein quantification, the Bradford reagent was diluted 1:5 in distilled water and a standard curve of bovine serum albumin (BSA) was

performed (0, 0.0625, 0.125, 0.25 and 0.5 mg/ml). In a multiwell plate, 10 µl of each sample (properly diluted) and standards reacted with 200 µl of Bradford reagent for 5 minutes. Then, absorbance was measured at 595 nm in an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). Obtained data allowed us to calculate protein concentration of each sample.

ELECTROPHORESIS AND TRANSFERENCE

The experimental samples included 15 µg of protein, loading buffer and distilled water until a volume of 20 µl. The mixture was heated for 20 minutes at 70ºC. Samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) gels at 60 V, while they were in the stacking gel (4,5% acrylamide), and 120 V during their migration in the separating gel (10% acrylamide). The Dual Color Precision Plus Protein Standard (Bio-Rad Laboratories, Hercules, CA, USA) was used as a molecular weight marker. Then, proteins were transferred onto 0.45 µm nitrocellulose membranes using a semi-dry transfer system TransBlot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) for 30 minutes at 25 V. Protein transfer was confirmed by staining nitrocellulose membrane with Ponceau S stain (Sigma, St. Louis, MO, USA) for 1 minute. Then, Ponceau S stain was washed three times with water and one time with TBS-T. Finally, the membrane was blocked with a blocking solution for 1 hour at room temperature.

IMMUNOBLOTTING

Immediately after membrane blockade, it was incubated overnight at 4°C with the suitable primary antibody properly diluted in blocking

solution. Four different primary antibodies were assayed. The information regarding the antigen detected by each antibody, the supplier reference of the antibody, the organism in which it was produced and the dilution of the primary and secondary antibodies is summarised in Table 7.

Antigen	Reference & Supplier	Organism	Dilution	Secondary dilution
GLT1	ab41621 Abcam	Rabbit	(1:5000)	(1:3000)
GLAST	NB100-1869 NovusBio	Rabbit	(1:2000)	(1:1000)
xCT	Ab175186 Abcam	Rabbit	(1:1000)	(1:1000)
GAPDH	G9545 Sigma-Aldrich	Rabbit	(1:2000)	(1:4000)
Rabbit IgG	#1706515 Bio-Rad	Goat		

Table 7. Characteristics of the different antibodies used in Western Blot procedure. The "reference and supplier" of each antibody and the "organism" they were obtained from are summarised. For primary antibodies the used dilution is indicated in the column "dilution". The used dilution for the secondary antibody is tabled in the last column for each primary antibody used.

After the overnight incubation, three washes in TBS-T 0.1% were performed before incubating the membranes with the secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature. The secondary antibody was removed, the membrane was washed again with TBS-T 0.1% three times and the membrane developing was performed. For that purpose, we used the enhanced chemiluminescence system Clarity Max ECL (Bio-Rad

Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Digital images of the immunoblots were obtained in a ChemiDoc Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Image analysis was performed with AlphaEaseFC software 4.0 (Alpha Innotech Corporation, San Leandro, CA, USA).

The intensity of the bands was expressed as arbitrary units and all of them were normalised with respect to GAPDH expression. The expression of each protein was analysed three times for each sample. The value for experimental groups was determined by establishing the control group (water exposed animals) to 100% and calculating the respective percentages, expressed as mean ± SEM.

4.1.4 EXPERIMENTAL DESIGN

Four experiments were designed to achieve the planned aims. According to the principles of the 3Rs stated by Russell and Burch in 1959 (replacement, reduction and refinement) (Russell & Burch, 1959) we tried to reduce the total number of animals in the present thesis by re-using the same animals in different experiments when appropriate.

Experiments 1 and 2 can be catalogued as **behavioural procedures**. They were carried out using male and female Wistar rats. Animals were exposed to chronic ethanol consumption under the ADE preclinical model. According to the obtained results detailed in section 5, only male animals from experiments 1 and 2 were used for the experiments 3 and 4. These experiments include **biochemical analysis**. Each experiment is detailed below.

4.1.4.1 Experiment 1. Identification and characterisation of two subpopulations of animals depending on their alcohol relapse-like drinking behaviour

Experiment 1 was designed to achieve **O1**. This experiment is divided in two sub experiments:

Experiment 1.A performed with 43 male Wistar rats exposed to the ADE model (described in section 4.1.3.1 and illustrated in Table 3) until week 32.

Experiment 1.B carried out with 38 female Wistar rats exposed to the ADE model (described in section 4.1.3.1 and illustrated in Table 4) until week 26.

In both cases, animals experienced three deprivation periods. According to the obtained results, two subpopulations were identified depending on their **manifestation or not of the ADE phenomenon**. After that, both subpopulations were characterised according to their ethanol consumption profile and preferences.

Identification of subpopulations

In order to study the relapse-like drinking behaviour displayed by our animals, the manifestation of the ADE phenomenon after each deprivation period was analysed. To do so, **basal ethanol intake** in each animal was determined by averaging the ethanol consumption values during the last three pre-abstinence days. The alcohol intake determined during the three days following the reintroduction of the ethanol bottles (**post-abstinence ethanol intake**) was also calculated.

According to the obtained results, animals were classified depending on their relapse-like drinking behaviour. The chosen criteria were:

— A **positive ADE** was considered when **ethanol consumption increased by more than 50%** with respect to the basal consumption determined before the deprivation period (Sinclair et al., 1973).

— Animals that, during the experiment, displayed **two or three positive ADEs** were assigned to the so-called "**ADE**" subgroup, while rats that expressed **only one or no ADE** episodes were assigned to the "**no-ADE**" subgroup.

A schematic representation of the stages in this protocol is depicted in Figure 11.

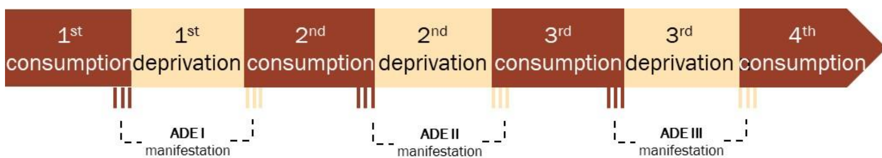


Figure 11. Schematic representation of consumption and deprivation periods in experiment 1. Vertical red lines represent the three pre-abstinence days used to obtain basal ethanol consumption while fair lines represent alcohol reintroduction days in which average ethanol consumption was calculated to obtain post-abstinence value.

Hence, after this analysis of the three deprivation periods, rats were classified according to their relapse-like behaviour as **ADE** or **no-ADE rats**.

Characterisation of subpopulations

Once the two subpopulations (ADE and no-ADE) were identified, their ethanol consumption behaviour was characterised. To this end, ethanol intake, total fluid intake, total ethanol preference and individual ethanol preference were determined for each subpopulation (see equations 1-4 in section 4.1.3.1).

These parameters, as well as rat weight (kg), were compared within and between subpopulations. In order to facilitate data comparisons, ethanol intake and ethanol preference were collapsed along each consumption period.

4.1.4.2 Experiment 2. Study of the efficacy of NAC in the prevention of alcohol relapse-like drinking behaviour.

This experiment was designed to achieve **O2.** Firstly, the entire cohort of animals underwent an additional alcohol deprivation period (4th deprivation period). Only the ADE rats that had repeatedly displayed the ADE phenomenon during experiment 1 were considered adequate to evaluate the pharmacological efficacy of NAC to prevent alcohol relapse. Hence, to carry out experiment 2, ADE rats were administered NAC or vehicle. All the pharmacological assays were initiated during the fourth alcohol deprivation period. Two sub experiments were performed:

Experiment 2.A was carried out with the **30 male ADE Wistar rats** that were randomly divided into three experimental groups:

- **ADE-Vehicle** (n=10): treated with vehicle (PB 0.4 M)
- **ADE-NAC 60** (n=10): treated with NAC 60 mg/kg
- **ADE-NAC 100** (n=10): treated with NAC 100 mg/kg

Experiment 2.B was performed with the **22 female ADE Wistar rats.** Due to the limited number of ADE rats in this experiment, only one NAC dose (60 mg/kg) was tested. The experimental groups were:

- **ADE-Vehicle** (n=11): treated with vehicle (PB 0.4 M)
- **ADE-NAC 60** (n=11): treated with NAC 60 mg/kg

Before the administration of the pharmacological treatment, all animals were handled to be habituated to the experimenter, the environment and the experimental procedure (subcutaneous injection).

They experienced a total of 5 five-minutes handling sessions. Rats were daily and subcutaneously injected with the adequate treatment two hours before the dark cycle started. The NAC doses and the administration schedule were selected according to previous studies (Corbit et al., 2014; Ducret et al., 2016; Quintanilla et al., 2016). In both experiments (2.A and 2.B) the 4th deprivation period lasted 21 days and the pharmacological treatment started 10 days before the reintroduction of the ethanol bottles. The total duration of treatment was 14 days: 10 doses were administered during the abstinence period and 4 doses during the alcohol reintroduction. The administration schedule is depicted in Figure 12.

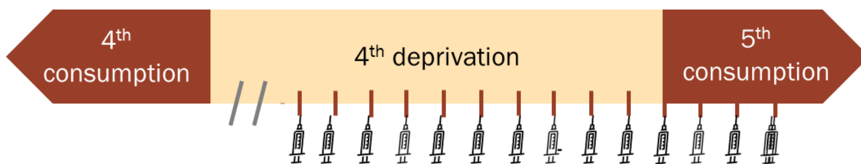


Figure 12. Schematic representation of the treatment schedule in experiment 2.A and 2.B. Animals received a daily subcutaneous dose of NAC or vehicle for 14 days: 10 under ethanol deprivation conditions and 4 after ethanol reintroduction.

In order to evaluate the anti-relapse efficacy of NAC, basal ethanol consumption and post-abstinence ethanol consumption were determined and compared within each experimental group. Moreover, total fluid intake and animal weight were controlled and compared before and after NAC treatment so as to explore the possible changes in water or food consumption patterns.

4.1.4.3 Experiment 3. Brain oxidative and neuroinflammatory status determined in male Wistar rats after 21 days of ethanol abstinence. Effects of alcohol reintroduction

This experiment was designed to achieve **O3**. The aim of this experiment was to explore whether the subpopulations identified in experiment 1 (ADE and no-ADE) differ in their brain oxidative and/or neuroinflammatory status after a prolonged ethanol abstinence (21 days). The effects of 24 hours of ethanol reintroduction were also explored in these animals. Experiment 3 was only performed with **male Wistar rats.** For the performance of this experiment, six different experimental groups were designed: Control, Ethanol, ADE abstinence, ADE reintroduction, no-ADE abstinence, no-ADE reintroduction.

Experimental groups

As previously explained, male rats from experiment 1 continued drinking and underwent a fourth deprivation period that lasted 21 days (see Table 3). At the end of this deprivation period no-ADE rats (n=13) were euthanised. Concretely, 6 of 13 were euthanised on the 21 day of ethanol abstinence (group **no-ADE abstinence**), and in 7 of 13 alcohol was reintroduced during 24 hours before being euthanised (group **no-ADE reintroduction**).

ADE rats that did not receive any NAC treatment during experiment 2 (i.e., animals that received vehicle injections) were considered adequate for experiment 3. Thus, these 10 ADE-Vehicle rats experienced a fifth ethanol deprivation period (5th deprivation period)

(see Table 3). 5 of them were sacrificed the 21st day of abstinence period (group **ADE abstinence**) and the rest were sacrificed after 24 hours of alcohol re-exposure (group **ADE reintroduction**). Figure 13 illustrates the moment when animals from each experimental group were sacrificed.

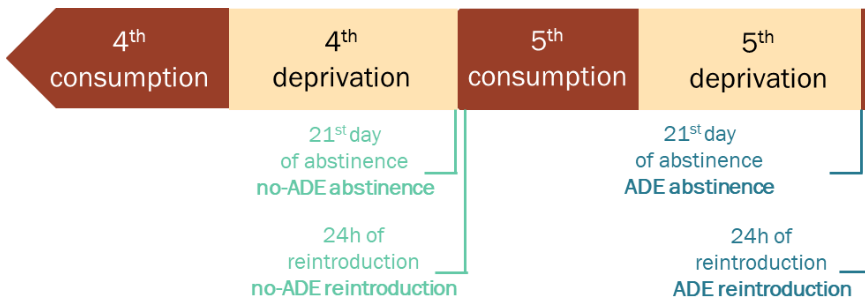


Figure 13. Schematic representation of the experimental design of experiment 3. No-ADE animals were sacrificed after the 4th deprivation period while ADE rats were sacrificed after the 5th deprivation period. In both subpopulations, animals included in the abstinence groups were sacrificed the 21st day of abstinence and animals from the reintroduction group were euthanised after 24 hours of alcohol reintroduction.

Two more groups of rats were planned in this experiment. On one hand, an additional nine male Wistar rats having free access to food and water that were never exposed to ethanol were also included. These animals served for testing if body weights of animals followed the same trend as that observed in ethanol-experienced rats and were also used as a **control group** for biochemical quantifications. On the other hand, six males were chronically exposed around five months to **ethanol** consumption having free access to food and four different bottles including tap water and ethanol dilutions (5, 10 and 20% v/v).

All in all, two different biochemical assays (corresponding to experiment 3.1 and 3.2) were performed in the six aforementioned experimental groups:

- **Control** (n=9): animals which were given continuous *ad libitum* access to tap water.
- **Ethanol** (n=6): animals which were given continuous *ad libitum* access to tap water and 5%, 10% and 20% ethanol dilutions
- **ADE abstinence** (n=5): ADE animals sacrificed under ethanol deprivation conditions
- **ADE reintroduction** (n=5): ADE animals sacrificed after 24 hours of ethanol reintroduction
- **no-ADE abstinence** (n=6): no-ADE animals sacrificed under ethanol deprivation conditions
- **no-ADE reintroduction** (n=7): no-ADE animals sacrificed after 24 hours of ethanol reintroduction

All animals (exposed to the ADE protocol or to chronic ethanol or water consumption) were euthanised to remove their brains that were dissected as explained in section 4.1.3.2.

EXPERIMENT 3.1. ANALYSIS OF OXIDATIVE STRESS STATUS

HIP and AMG are brain areas strongly affected by ethanol consumption and abstinence, situations that could cause an imbalance in redox status (Elibol-Can et al., 2011; Roberto et al., 2004). For this reason, in the present thesis we explored if ethanol abstinence and reintroduction differently affect oxidative stress levels in these areas in the two identified subpopulations, ADE and no-ADE rats.

Tissue from HIP and AMG was analysed by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). With this technique it was possible to determine GSH and GSSG content in brain tissue. The procedure was detailed in 4.1.3.3.

EXPERIMENT 3.2. DETERMINATION OF NEUROINFLAMMATORY MEDIATORS

Ethanol consumption triggers inflammatory processes in the brain. Particularly, inflammatory processes in cortex have been described in alcoholic humans and rodents (Vetreno et al., 2013; Zhao et al., 2013). In accordance with this idea, we proposed to study the expression of several proinflammatory markers in PFC in the two identified subpopulations. This study was performed after a protracted abstinence or after ethanol reintroduction. Concretely, the mRNA expression of IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3 (Crews & Vetreno, 2016; Fernandes et al., 2017; Kelley et al., 2019) was analysed by RT-PCR technique, according to the procedure detailed in section 4.1.3.4.

4.1.4.4 Experiment 4. Unravelling the mechanism of action underlying NAC anti-relapse effect.

This experiment was carried out to achieve **O4.** Once the effect of NAC on the prevention of ethanol relapse was demonstrated under our experimental conditions (experiment 2 of the present thesis), the next step was to explore its potential mechanism of action. According to the pharmacological properties conferred to NAC (see section 2.4.2) experiment 4 was designed to explore whether NAC modulates **oxidative stress, neuroinflammation and glutamatergic homeostasis** during ethanol abstinence (McClure et al., 2014; Smaga et al., 2021). This set of experiments was performed in a cohort of male Wistar rats

Experimental groups

Experiment 4 was performed with 15 **ADE male Wistar rats** proceeding from experiment 2. These animals underwent an additional ethanol deprivation period (5th deprivation period). Each rat received the same treatment that it had previously received in experiment 2. Accordingly, three experimental groups were designed:

- **ADE Abs-Vehicle** (n=5): received vehicle (PB 0.4 M)
- **ADE Abs-NAC 60** (n=5): received NAC 60 mg/kg
- **ADE Abs-NAC 100** (n=5): received NAC 100 mg/kg

Animals were handled to be habituated to the experimenter, the environment and the experimental procedure (subcutaneous injection). All animals were exposed to five handling sessions of five minutes before the treatment started.

The pharmacological treatment in experiment 4 followed a similar schedule to experiment 2. Nonetheless, rats were sacrificed during the abstinence period. Consequently, they received only 10 doses of treatment instead of 14. In summary, it must be considered that in this experiment:

- The ethanol deprivation period lasted 21 days.
- The pharmacological treatment began 10 days before the end of the deprivation period.
- Each animal received the same treatment as in experiment 2.
- Rats received one daily subcutaneous injection of treatment two hours before the dark cycle started. They received a total of 10 doses.
- Animals were sacrificed in abstinence 24 hours after the last dose.

A schematic representation of the treatment schedule of experiment 4 is depicted in Figure 14.

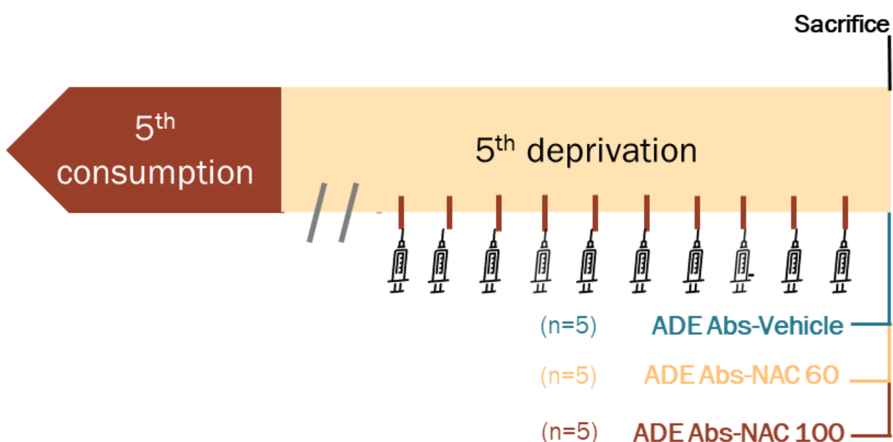


Figure 14. Schematic representation of treatment schedule in experiment 4. Animals were sacrificed under abstinence conditions 24 hours after the last dose of vehicle (n=5), NAC 60 mg/kg (n=5) or NAC 100 mg/kg (n=5).

Apart from these 15 animals, **control** and **ethanol** groups from experiment 3 were also included in the biochemical procedures of experiment 4.

Finally, it should be noticed that the ADE abstinence group from experiment 3 is the same group as the **ADE Abs-Vehicle** from experiment 4. Nomenclature has been changed to adapt the group name to each experiment. All in all, Table 8 has been prepared so as to facilitate the traceability of each male Wistar rat used in experiments from 1 to 4.

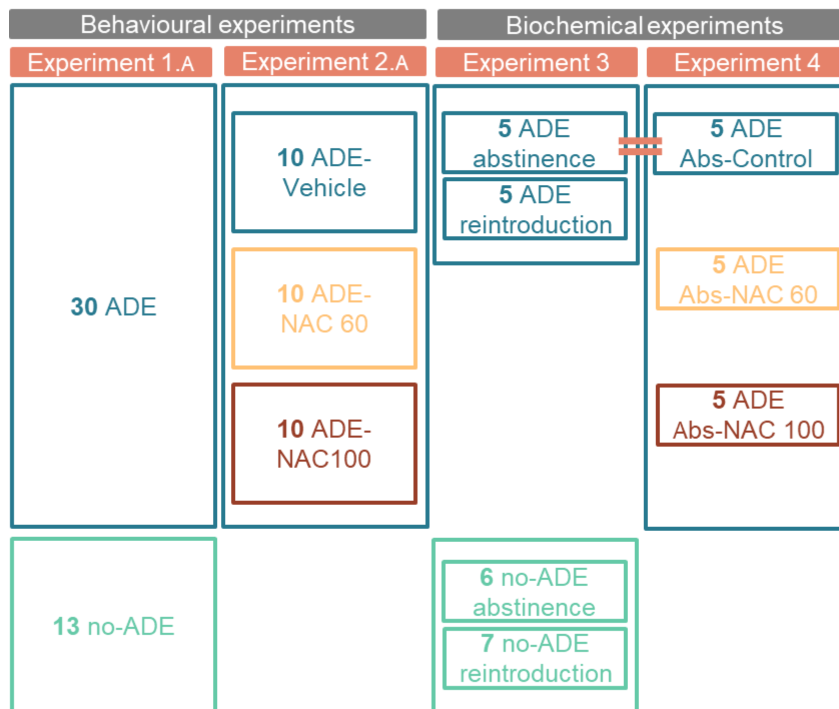


*Table 8. Traceability of each **male Wistar rat** used in experiments 1 to 4. Colours and names assigned to each group are maintained during all the thesis. Bold numbers refer to the sample size of each group. Red equals signs means that both groups were composed exactly by the same animals although they were renamed for experiment 4.*

Biochemical analysis

In order to explore the potential mechanism that underlies the ethanol anti-relapse effect of NAC, three biochemical assays were performed:

Experiment 4.1. Effect of NAC treatment on oxidative stress status during abstinence.

Experiment 4.2. Determination of neuroinflammatory mediators after NAC administration during ethanol deprivation period.

Experiment 4.3. NAC effect on the expression of glutamatergic proteins under abstinence conditions.

As in experiment 3, oxidative stress status was determined in HIP and AMG and the expression of different neuroinflammatory mediators in PFC was studied. Moreover, there is evidence of augmented levels of glutamate in striatum during ethanol abstinence (Alasmari et al., 2018). Hence, the potential effects of NAC in the striatal glutamatergic homeostasis during ethanol abstinence were also analysed. Tissues from DS and nucleus accumbens were properly processed in order to study the expression of GLT1, GLAST and xCT proteins as detailed in section 4.1.3.5.

4.1.5 Statistical analysis

All data are presented in this thesis as mean ± standard error of mean (SEM). Statistical analysis confidence level was adjusted to 95%, hence significant differences were considered when p-value was less than 0.05. Assumptions of normality and homoscedasticity were checked before performing the definitive statistical test. In addition, the Box-Pierce test was applied when mixed two-way ANOVA for repeated measures was intended to be used.

Analyses were carried out by using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA) and IBM SPSS statistics V.26 (IBM Corp, Armonk, New York, USA).

4.1.5.1 Experiments 1 and 2

Experiments 1 and 2 were focused on the analysis of several ethanol consumption parameters. Additionally, the manifestation of the ADE phenomenon was evaluated through the comparison between basal and post-abstinence ethanol intake by **paired Student's t-test.**

In experiment 1, ethanol intake and ethanol preference at each deprivation period was analysed through **a mixed two-way ANOVA for repeated measures** with time being the within-groups factor and subpopulation the between-groups factor.

Ethanol intake and ethanol preference between ADE and no-ADE subpopulations along the four consumption periods experienced was

analysed using a **mixed two-way ANOVA**, with consumption period being the within-groups factor and subpopulation the between-groups factor.

Particular ethanol-dilution preferences between the ADE and no-ADE groups along months were analysed through **a three-way ANOVA** with months being the within-groups factor and ethanol dilution and subpopulation the between-groups factors.

Lastly, the comparison in body weight and total fluid intake between the ADE and no-ADE groups was analysed through a **multiple t-test.**

In experiment 2, ethanol intake along 8 days (4 days before and after the abstinence period) was analysed both by a **one-way repeated-measures ANOVA**, time being the targeted factor, and by **a paired Student's t test.** In this latter comparison, the daily alcohol intake obtained on the 4 days before and after the abstinence period was collapsed in one mean value. When significant differences were detected, a **multiple comparison Tukey test** was applied.

Body weight and total fluid intake before and after NAC treatment were analysed through a **paired Student's t-test.**

4.1.5.2 Experiments 3 and 4

Experiments 3 and 4 were focused on the analysis of different biochemical and molecular parameters.

In experiment 3, levels of GSH, GSSG, GSSG/GSH and mRNA expression (IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3) were analysed using **one-way ANOVA.** Additionally, a **two-way ANOVA** was also performed with subpopulation and alcohol reintroduction being the

factors analysed. When significant differences were found, a *post-hoc* **Tukey 's test** was performed.

In experiment 4, experimental data, i.e., levels of GSH, GSSG, GSSG/GSH, mRNA expression (IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3) and protein expression (GLT1, GLAST and xCT) were analysed using two different analyses of variance of one factor (**one-way ANOVA**). The influence of ethanol consumption or ethanol abstinence was studied through the comparison among data derived from Control, Ethanol and ADE Abs-Vehicle group. The comparison among data from ADE Abs-Vehicle, ADE Abs-NAC 60 mg/kg and ADE Abs-NAC 100 mg/kg allowed us to explore the effect of NAC under abstinence conditions. *Post-hoc* comparisons were performed through the application of **Tukey's test** when appropriate.

4.2. Acute effects of NAC in the MCLS

For the study of the acute effects of NAC in the MCLS, three experiments were performed:

— Experiment 5. Acute effect of NAC onto the MCLS activation

— Experiment 5.1. The efficacy of MTEP to suppress the activation of MCLS induced by NAC.

— Experiment 5.2. The effect of 120 mg/kg of NAC on the ethanol-induced activation of the MCLS

The experimental procedures under the design of each experiment are detailed in the following sections.

4.2.1 Animals

Experiment 5 was carried out in the Central Service for Experimental Research Support (SCSIE) of the University of Valencia. 58 male Wistar rats were purchased from Envigo (Barcelona, Spain). Animals were housed in groups of 3-4 individuals. Rats weighed 312 ± 27 g at the beginning of the experiment. Housing conditions and protection policies were the same as previously explained in section 4.1.1. The authorised procedures references were: 2017/VSC/PEA/00086 and 2020/VSC/PEA/0020.

4.2.2 DRUGS

Ethanol was purchased as 96% v/v ethanol from Scharlau S.A (Madrid, Spain). For intra-VTA microinjections, 150 nmol of ethanol were diluted in artificial cerebrospinal fluid (aCSF) and pH was adjusted to 6.5.

MTEP (3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine) is a selective and non-competitive antagonist of the metabotropic glutamate receptor 5 (mGluR5). It was supplied by Sigma-Aldrich (Saint Louis, MO, USA). Its molecular weight is 200.26 g/mol and its chemical structure is shown in Figure 15. For its intraperitoneal administration, it was dissolved in normal saline at a concentration of 0.1 mg/ml.

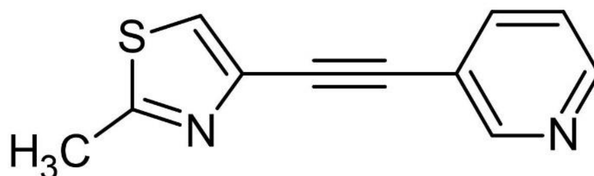


Figure 15. Chemical structure of MTEP

NAC ($C_5H_9NO_3S$) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). For its intraperitoneal injection, it was freshly dissolved in PB 0.4 M to a final concentration of 15 or 60 mg/ml. pH was adjusted with NaOH to 7.4.

4.2.3 Experimental procedures

4.2.3.1 Surgical and post-surgical care

For performing experiment 5.2 (see section 4.2.4) the stereotaxic implantation of an **intra-VTA guide cannula** was required.

Rats were intraperitoneally anaesthetised with ketamine hydrochloride/xylazine hydrochloride (95 mg/kg of ketamine and 10 mg/kg of xylacine) (Esteve veterinaria, Barcelona, Spain) and placed in a stereotaxic apparatus (Stoetling, USA). The skin was disinfected with povidone-iodine complex solution (10%) and chlorhexidine 20% solution before making an incision (8–10 mm) in the skin over the skull. The resulting wound was infiltrated with lidocaine (3%). Then, two holes were drilled: one for the skull screw and the other for the guide cannula (Plastics One, Roanoke, VA, USA).

Each animal was implanted unilaterally with a 28-gauge guide cannula aimed at 1.0 mm above the posterior VTA. The coordinates relating to the bregma and the skull surface (Paxinos & Watson, 2007) were as follows: anteroposterior -5.8 mm; mediolateral -2.1 mm; dorsoventral -8.1 mm. Cannulae were angled toward the midline at 10º from the vertical plane (all the measurements in the dorsal–ventral plane refers to distances along the track at 10º from the vertical plane). The cannula assemblies were secured in place with reinforced glass cement (GC FujiCEM®, GC corporation, Tokio, Japan). 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.

Following surgery, the rats were housed in individual rectangular plastic cages (42.5x20x14 cm^3) located side by side in order to prevent the influence of chronic stress due to isolation on performance. They had free access to food and water for at least 7 days.

4. 2.3.2 Handling

For the indirect evaluation of the MCLS activation (experiment 5.1 and 5.2), animals were subjected to a previous handling to accustom to the experimenter, the experimental room, and to the i.p. and intra-VTA injection procedure. Animals received a total of four to seven handling sessions to decrease the activating effects associated with the manipulations taking place during the injection process, as well as the novelty activating effects of the testing room. For each handling session each animal was taken from the colony, brought to the experimental room, and handled for 5 minutes per day by the researcher. In the case of experiment 5.2, handling sessions started two days after surgery and were performed until the experimental day. Animals also experienced a simulation of a VTA-microinjection by removing the cannula lid in each session.

4.2.3.3 Immunohistochemistry of cFos

In order to study cell activation in nucleus accumbens, immunohistochemistry against the cFos transcription factor, an immediate early gene traditionally used as an indicator of neural activation (Kovács, 2008), was performed in experiment 5.1 and 5.2. **cFos** is the most widely used functional anatomical marker of activated neurons within the central nervous system: at basal condition it is

expressed at low levels all over the brain; several extracellular signals have been shown to induce its expression; cFos detection can be easily carried out and combined with other markers (Jaworski et al., 2018)

WORKING SOLUTIONS

- **aCSF** was used as a vehicle for intra-VTA microinjections. Its composition was: 120.0 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 100 mM D-glucose, and 0.2 mM ascorbate.

The following solutions were used for the intracardiac perfusion and to fix the tissue:

- **PB 0.4M** was prepared and stored at room temperature. One litre contains: 13.8 g of sodium phosphate dihydrate, 42.58 g of disodium phosphate in distilled water. pH was properly adjusted to 7.4.
- **Phosphate buffer saline (PBS)** was prepared at 10X concentration and stored at room temperature: 3.9 g of disodium phosphate dihydrate, 10.65 g of disodium phosphate and 83 g of sodium chloride in distilled water until 1 litre. The final concentration of PBS (1X) was prepared before each use by mixing 100 ml of PBS 10X and 900 ml of distilled water.
- **Formaldehyde (FA)** at 4% is used to fix tissues during perfusion. It was freshly prepared before use: 114 ml of FA 40% were mixed with 250 ml of PB 0.4M and 636 ml of distilled water.
- **Sucrose 30%** in PB was dissolved to be used as tissue cryoprotective agent for brain storage. 300 g of sucrose were

mixed with 250 ml of PB 0.4M and diluted until 1 litre in distilled water.

For the immunohistochemical determination of cFOS, four trizma-based solutions were prepared and stored at 4ºC:

- One litre of **Tris 0.5 M** in distilled water contained 60.55 g of trizma base. **Trizma buffer (TB) 0.05M** was prepared as 100 ml of tris 0.5 M and 900 ml of distilled water. pH was adjusted to 7.6.
- To prepare **Trizma buffer saline (TBS)** 9 g of sodium chloride were diluted in one litre of TB 0.05M. Lastly, by adding 10 ml of triton-Tx 100 to 90 ml of TBS, **(TBS-Tx100 10%)** was obtained. 0.015 ml were mixed with 5 ml of TBS to prepare **TBS-Tx100 0,3%.**
- To mount sections on the slides**, gelatine 0.2%** in TB was used. 0.2 g of gelatine were diluted in 100 ml of TB 0.05M and 500 μl of 4% p/v of sodium azide were added.
- To stain sections, **cresyl violet** was prepared by adding 1.25 g of cresyl violet (Sigma, St. Louis, MO, USA) to 50 ml of ethanol 96º. The mix was agitated overnight and filtered. Then, 200 ml of distilled water was added.

INTRACARDIAC PERFUSION AND TISSUE COLLECTION

Rats were deeply anaesthetised with pentobarbital (40 mg/kg) and intracardially perfused with 200 ml of PBS, followed by 300 ml of FA 4% in PB 0.1 M. Then, the brain was removed, and kept in FA 4% solution for 20 hours at 4 ° C. Then, it was transferred to a 30% sucrose

solution for a minimum of 4 days at 4 °C. Finally, 40 µm brain sections were obtained using a microtome (Leica, Nussloch, Germany) and they were collected in 30% sucrose solution (Zornoza et al., 2005). Samples were stored at -20ºC until they were used.

cFos Immunohistochemistry

Before use, brain sections were washed three times for 5 minutes with TBS to remove sucrose. Sections including nucleus accumbens were selected according to coronal plates from Rat Brain Atlas (Paxinos & Watson, 2007). Figure 16 shows the brain selected sections and the defined areas for the quantification of the expression of cFOS protein.

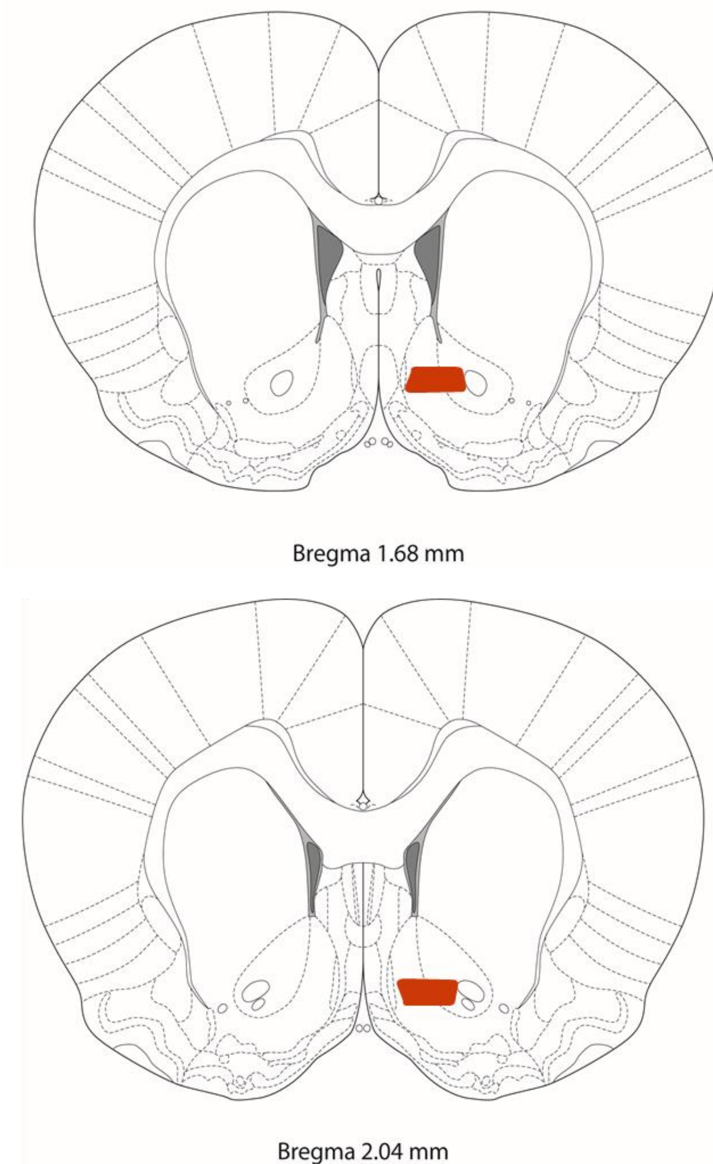


Figure 16. Brain sections selected for cFOS immunohistochemistry from Paxinos and Watson, 2007. Red marks represent the area of study where cFOS-IR cells were analysed.

Then, sections were incubated in 1% hydrogen peroxide in TBS for 30 minutes to block the activity of endogenous peroxidase present in the tissue. Next, sections were washed with TBS for 15 minutes and then blocked with 5% normal goat serum in TBS-Tx100 0.3% during one hour. Following, sections were incubated in the presence of an anti-cFOS polyclonal antibody (1:20000 in TBSTx100 0,3%; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4ºC. The following morning, sections were washed in TBS before being incubated with a biotinylated anti-rabbit antibody (1:200 in TBSTx100 0,3%; Vector Labs) for 2 hours at room temperature. After washing sections, they were incubated with an avidin-biotinylated peroxidase complex (1:200; ABC Elite Kit; Vector Laboratories, Inc., Burlingame, CA, USA) for 90 minutes at room temperature. The reaction was visualised by incubating with a commercial kit containing 1% diaminobenzidine solution. (SigmaFAST, Sigma, St. Louis, MO, USA), after 30 minutes the reaction was stopped by transferring sections to TB. Finally, the sections were embedded in a 2% gelatine solution, mounted on slides and dried. The following days sections were dehydrated in alcohols (2x5 min in ethanol 96º, 2x5 min in ethanol 100º and 2x5 min in xylol), cleared, and coverslipped with Eukitt (Sigma, St. Louis, MO, USA) for microscopical examination.

INJECTOR PLACEMENT VALIDATION

Animals from experiment 5.2 were microinjected in the VTA through a 33-gauge stainless steel injector. To verify that the injector position was correct, brain slices from VTA were stained with cresyl violet. To do so, sections were mounted on slides with 0.2% gelatine in TB and incubated for 5 minutes in cresyl violet. Posteriorly, they were washed

three times with distilled water before being treated for 3.5 minutes with ethanol 96° with 0.2% of acetic acid. Finally, three more washes with distilled water were performed. The placement of the tip of the microinjection cannula in the VTA was determined and finally validated according to the rat brain atlas (Paxinos & Watson, 2007).

IMAGE ANALYSIS

The number of **cFOS-immunoreactive cells (cFOS-IR cells)** was determined in nucleus accumbens (Figure 16). Four sections per animal were selected, and images were digitised using a microscope (Leica) equipped with a CCD camera. The 10X objective was selected to obtain a frame of 1026 x 769 mm. The counting of the stained nuclei per frame was carried out using the Multipoint plugin of the ImageJ software (NIH, USA). Two experimenters performed simultaneously the image counting. Both researchers were blind to experimental grouping throughout the image acquisition and processing.

4.2.3.4 DRUG VTA-MICROINJECTION PROCEDURE

According to the literature, an injection of **150 nmol of ethanol** in the posterior VTA (pVTA) is able to induce the activation of the MCLS (Martí-Prats et al., 2010; Sánchez-Catalán et al., 2009). All the intra-VTA drug microinjections were carried out using a 33-gauge stainless steel injector, extending 1.0 mm below the tip of the guide cannula. The injector was attached to a 25 µl Hamilton syringe using PE-10 tubing. All microinjections were carried out using a syringe pump (Kd Scientific Inc, Holliston, MA, USA), which was programmed to deliver a total volume of 200 nl in 20 s (flow rate of 0.6 µl/min). Following the

infusion, the injector remained in place for 1 min to allow the diffusion of the drugs; then, it was removed and the lid was replaced. All the injections were administered in the experimental room. This procedure, extensively validated in our laboratory (Martí-Prats et al., 2010; Sánchez-Catalán et al., 2009), was used to perform experiment 5.2.

4.2.3.5 Locomotor activity test

In experiment 5.2, the activation of the MCLS was indirectly explored through the measurement of the locomotor activity displayed by rats.

Tests were performed 7 days after surgery. The day before the experiment, the rats were taken from the colony room, brought to the experimental room, and placed, for 30 minutes, in the same type of rectangular cages in which the animals were housed (42.5x20x14 cm^3). The day of the experiment, after receiving the planned treatments, rats were placed in their experimental cage. The distance travelled in 20 minutes (expressed in cm) by each animal was recorded by a digital video camera in order to quantify the locomotor activity. To this end, the experimenter counted the number of crossings and 360º rotations performed by the animal during 20 minutes. Then, the total number of crossings and rotations was multiplied by the length of the cage to calculate the total distance travelled by each animal.

4.2.4 EXPERIMENTAL DESIGN

4.2.4.1 EXPERIMENT 5. ACUTE EFFECT OF NAC ONTO THE MCLS ACTIVATION

This experiment was carried out to achieve **O5.** It was designed to study the acute effects of NAC onto the MCLS. Two experiments were designed:

- In experiment 5.1, we analysed **the acute effect of two different systemic doses of NAC on the activation of nucleus accumbens neurons.** It was evaluated through the quantification of cFOS-IR cells expression in this brain area of the rats. The influence of MTEP, a selective negative allosteric modulator of mGluR5, in the activation induced by NAC was also explored.
- In experiment 5.2, we explored the effects of the administration of a **high NAC dose (120 mg/kg) on the neurochemical and behavioural activation of the MCLS**, which was induced via the intra-VTA administration of ethanol.

EXPERIMENT 5.1. THE EFFICACY OF MTEP TO SUPPRESS THE ACTIVATION OF MCLS INDUCED BY NAC.

Experiment 5.1 was designed to address the following questions:

Is NAC able to activate the MCLS depending on the administered dose? To answer this question, two acute different doses of NAC were i.p. administered: 30 mg/kg (low dose) or 120 mg/kg (high dose).

Does the mGluR5 receptor participate in the activating effects of NAC onto the MCLS? To clear this up, the antagonist of the mGluR5 receptor, MTEP, was co-administered in the presence or absence of NAC.

Five days before the experimental day, 34 male Wistar rats were handled daily (see section 4.2.3.2). Then, rats were randomly assigned to one of the six experimental groups detailed in Table 9. This table gathers the name of each experimental group, the group size (n) and the administered treatments.

Group	n	Treatment i.p. 1	Treatment i.p. 2
Veh/Sal	4	Saline	Vehicle
NAC 30/Sal	6	Saline	NAC 30 mg/kg
NAC 120/Sal	6	Saline	NAC 120 mg/kg
Veh/MTEP	6	MTEP 0.1 mg/kg	Vehicle
NAC 30/MTEP	6	MTEP 0.1 mg/kg	NAC 30 mg/kg
NAC 120/MTEP	6	MTEP 0.1 mg/kg	NAC 120 mg/kg

Table 9. Experimental groups of experiment 5.1. Group size (n) and the two administered treatments are consigned for each experimental group. In the first i.p. treatment animals received saline or MTEP 0.1 mg/kg. In the second i.p. treatment animals received vehicle, NAC 30 mg/kg or NAC 120 mg/kg.

Each rat received two consecutives intraperitoneal (i.p.) injections. Injection 1 included **saline or MTEP 0.1 mg/kg** and injection 2 included **vehicle (PB 0.4M), NAC 30 mg/kg or NAC 120 mg/kg.** MTEP dose was

selected according to previous research (Kupchik et al., 2012). Two hours after the i.p. injections, rats were deeply anaesthetised with pentobarbital 40 mg/kg and intracardially perfused. Brains were collected and the activation of the MCLS was analysed through the quantification of cFOS-IR cells in the nucleus accumbens (see section 4.2.3.4). An overview of the timeline is depicted in Figure 17.

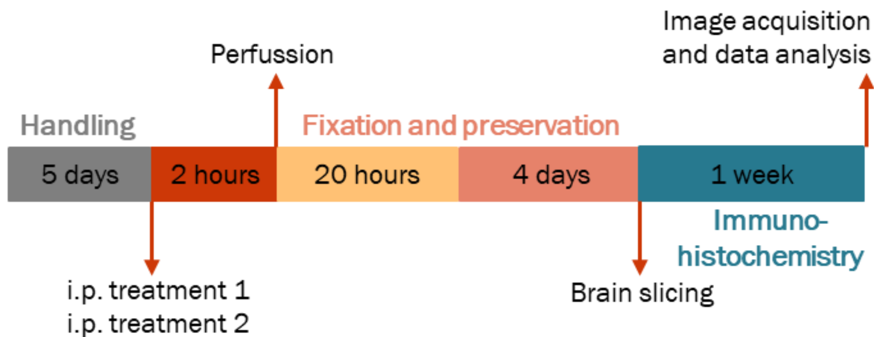


Figure 17. Timeline of experimental procedure in experiment 5.1

Experiment 5.2. The Effect of 120 Mg/kg of NAC on the Ethanol-Induced Activation of the MCLS

Previous results obtained in experiment 2 demonstrated the anti-relapse effect of NAC when alcohol is reintroduced after a period of abstinence. Thus, experiment 5.2 was designed to gain an insight into the ethanol anti-relapse effect of NAC. In order to do so, the aim of this experiment was to determine whether NAC would be able to attenuate or suppress the reinforcing effects of ethanol triggered by its ability to activate the MCLS.

Specifically, 120 mg/kg of NAC (60 mg/ml) were i.p. injected and 150 nmol of ethanol were intra-VTA microinjected. As previously explained, the selected dose of ethanol ensures the activation of the MCLS under our experimental conditions (Martí-Prats et al., 2010; Sánchez-Catalán et al., 2009). Besides, the NAC dose was selected according to previous preclinical assays (Laverde et al., 2021; Quintanilla et al., 2018). The activation of the MCLS was indirectly measured by analysing the locomotor activity displayed by the animal, and directly measured through the evaluation of cFOS expression.

24 male Wistar rats had been implanted with an intra-VTA cannula (explained in section 4.2.3.1). Two days after, they were handled daily for five more days. Rats were randomly assigned to four experimental groups depending on the i.p. and intra-VTA treatment as detailed in Table 10.

Group	n	Treatment i.p.	Intra-VTA microinjection
aCSF/Sal	6	Saline	aCSF
aCSF/ NAC 120	6	NAC 120 mg/kg	aCSF
EtOH/Sal	6	Saline	Ethanol 150 nmol
EtOH/ NAC 120	6	NAC 120 mg/kg	Ethanol 150 nmol

Table 10. Experimental groups of experiment 5.2. Group size (n) and treatments are detailed for each group. In the i.p. treatment animals received saline or NAC 120 mg/kg. In the intra-VTA treatments animals received aCSF of ethanol 150 nmol. The intra-VTA microinjections were administered 30 minutes after the i.p. injection.

The day of the experiment, rats were placed in their own experimental cage and were i.p. injected with **saline or NAC 120 mg/kg.** 30 minutes later **150 nmol of ethanol or aCSF** were intra-VTA microinjected according to section 4.2.3.3.

Immediately after the microinjection, the locomotor activity test was performed as previously explained (section 4.2.3.5). Finally, two hours after the intra-VTA injections rats were deeply anaesthetised and intracardially perfused. Brains were collected and the activation of the MCLS was analysed through the quantification of cFOS-IR cells in the nucleus accumbens (see section 4.2.3.3). A procedure overview is depicted in Figure 18.

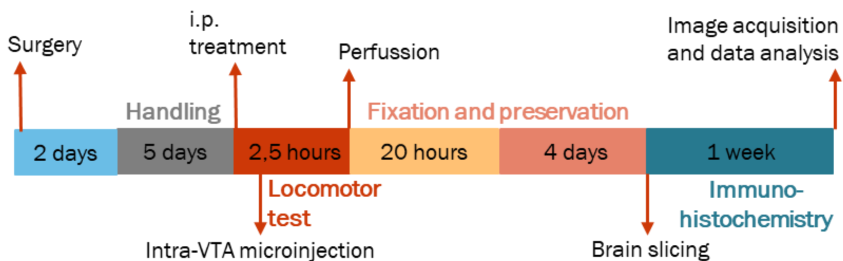


Figure 18. Timeline of experimental procedure in experiment 5.2

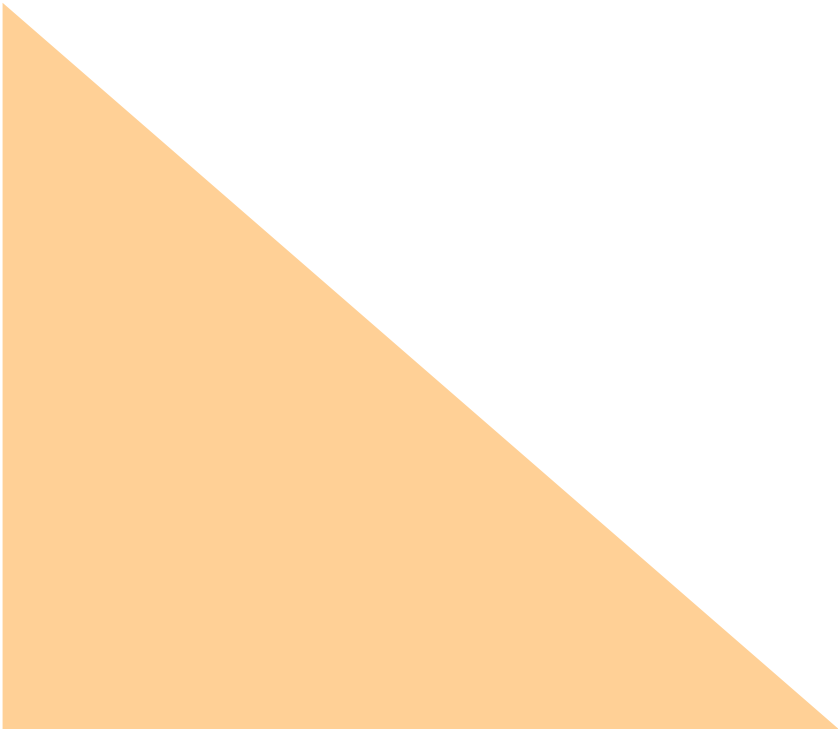
4.2.5 STATISTICAL ANALYSIS AND SOFTWARE

All the data presented in this thesis is expressed as mean ± standard error of the mean (SEM). In every statistical analysis the confidence level was adjusted to 95%, hence significant differences were found when p-value was less than 0.05.

Graphs and statistical analysis were performed with GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA).

In experiment 5.1 and 5.2, after testing for normality with the **Shapiro–Wilk test**, the number of cFOS-IR cells and total distance travelled were analysed using a **one-way ANOVA**, followed by **Tukey's test**, if applicable. Homogeneity of variance was tested before the ANOVA was performed. In cases where the normality test failed, a non-parametric **Kruskal–Wallis test** was performed.

5. RESULTS



5.1. Study of the neurobiology of abstinence and relapse to alcohol consumption. Evaluation of N-acetylcysteine as a potential anti-relapse pharmacotherapy

5.1.1 Experiment 1: Identification and characterisation of two subpopulations depending on their relapse-like drinking behaviour

Rats were subjected to a long-term voluntary ethanol drinking procedure with repeated deprivation periods. Based on the previous explained criteria (4.1.4.1), experiment 1 allowed us to categorise our rats according to their relapse-like drinking behaviour. This categorisation was performed once rats had experienced three deprivation periods. Rats that expressed 2 or 3 positive ADE were assigned to the ADE group while rats that expressed one or none ADE were assigned to the no-ADE group.

5.1.1.1 Experiment 1.A. Male relapse-like drinking behaviour determined under the ADE protocol

According to the obtained results, 30 animals fitted with the **ADE** group criteria while 13 animals were assigned to the **no-ADE** group.

In Figure 19, the average ethanol intake displayed, during the course of the experiment 1.A, by both experimental groups is plotted.

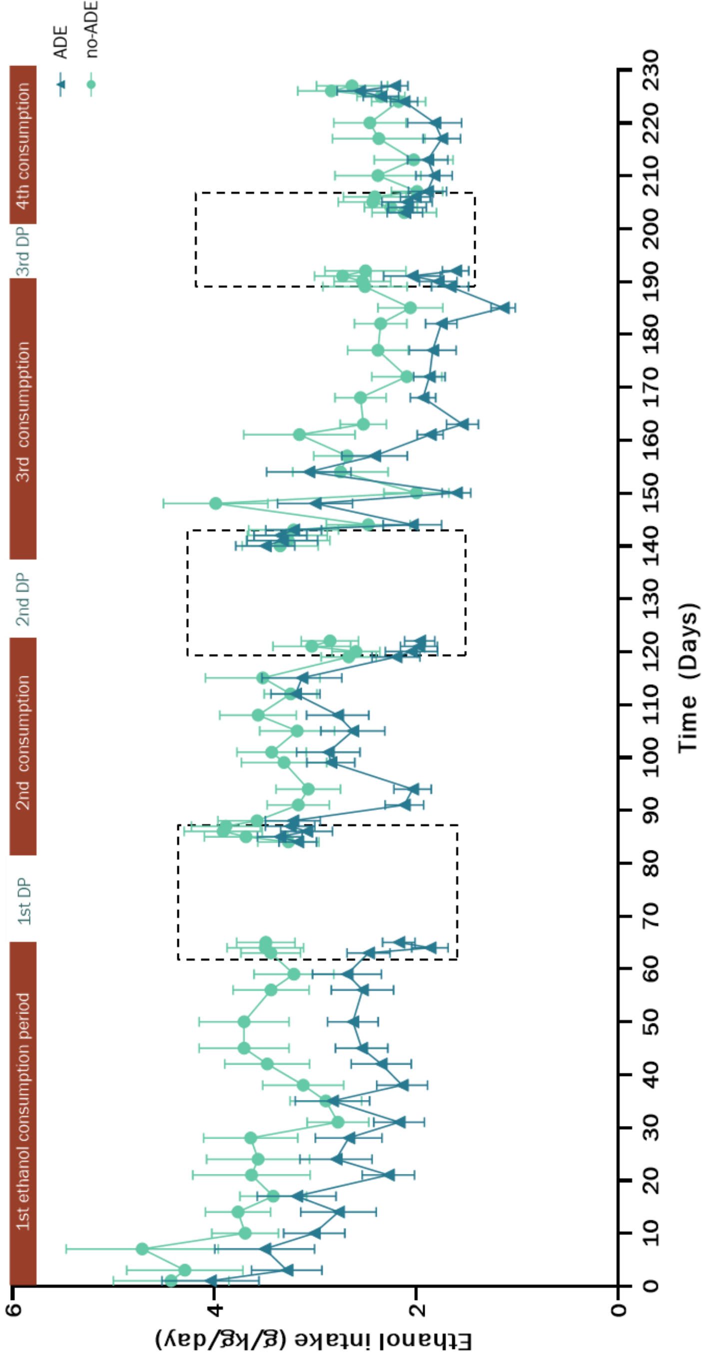
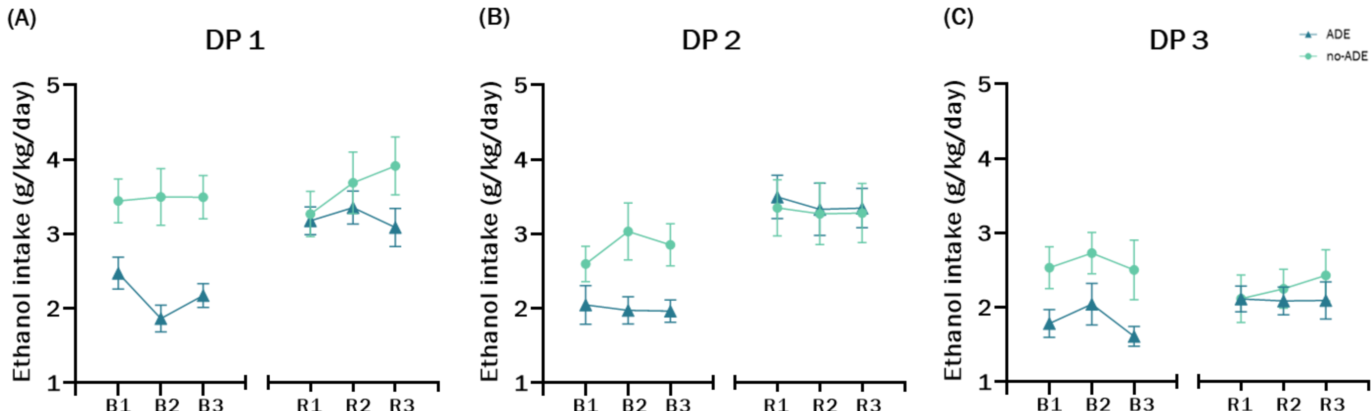


Figure 19. Average voluntary **ethanol intake** (expressed in g/kg/day) displayed by rats categorised in the **ADE** (blue triangles, n=30) or **no-ADE** (green circles n=13) groups along the entire experiment. Dashed-line rectangles indicate the pre- and post-abstinence values at each period of deprivation. Data are represented as mean ± SEM. DP: deprivation period.

As can be observed in Figure 19, the voluntary ethanol intake along time seemed to be **different between the two subpopulations.** On most days, ADE rats displayed a lower ethanol consumption than no-ADE animals. Data included in each dashed-line rectangle were used to perform a two-way ANOVA for repeated measures with time being the within-group factor and subpopulation the between-group factor. Ethanol intake along the three days before and after each deprivation period displayed by both subpopulations (ADE and no-ADE) was compared.

The mean ethanol intake values included in this analysis and the statistical results obtained through the two-way ANOVA are plotted in Figure 20.



(A)

Time	[F (5, 205)=8.930; p<0.001]
Subpopulation	[F(1, 41)=7.150; p=0.011]
Interaction	[F(5, 205)=6.108; p<0.001]

(B)

Time	[F(5, 205)=10.628; p<0.001]
Subpopulation	[F(1, 41)=1.096; p=0.301]
Interaction	[F(5, 205)=2.839; p=0.017]

(C)

Time	[F (5, 205)=0.430; p=0.827]
Subpopulation	[F(1, 41)=5.855; p=0.020]
Interaction	[F(5, 205)=2.542; p=0.029]

Figure 20. On the top: ***Ethanol intake*** *(expressed in g/kg/day) displayed by ADE (blue triangles, n=30) and no-ADE animals (green circles, n=13) during the days* ***before*** *(B1, B2 and B3)* ***and after*** *(R1, R2 and R3)* ***each deprivation period:*** *panel (A) includes data derived from the 1st deprivation period (DP1), panel (B) includes data derived from the 2nd deprivation period (DP2) and panel (C) includes data derived from the 3rd deprivation period (DP3).* Data are represented as mean ± SEM. DP: deprivation period. On the bottom: Statistical results from **two-way ANOVA** analysis of the depicted data (time x subpopulation).

As can be observed in Figure 20, statistical analysis identified the existence of **significant time and subpopulation effects, but also an interaction** between both factors in all situations. Accordingly, a paired Student's t-test, collapsing data before and after each deprivation period, was also performed. Collapsed data used to perform additional statistical analysis are depicted in Figure 21.

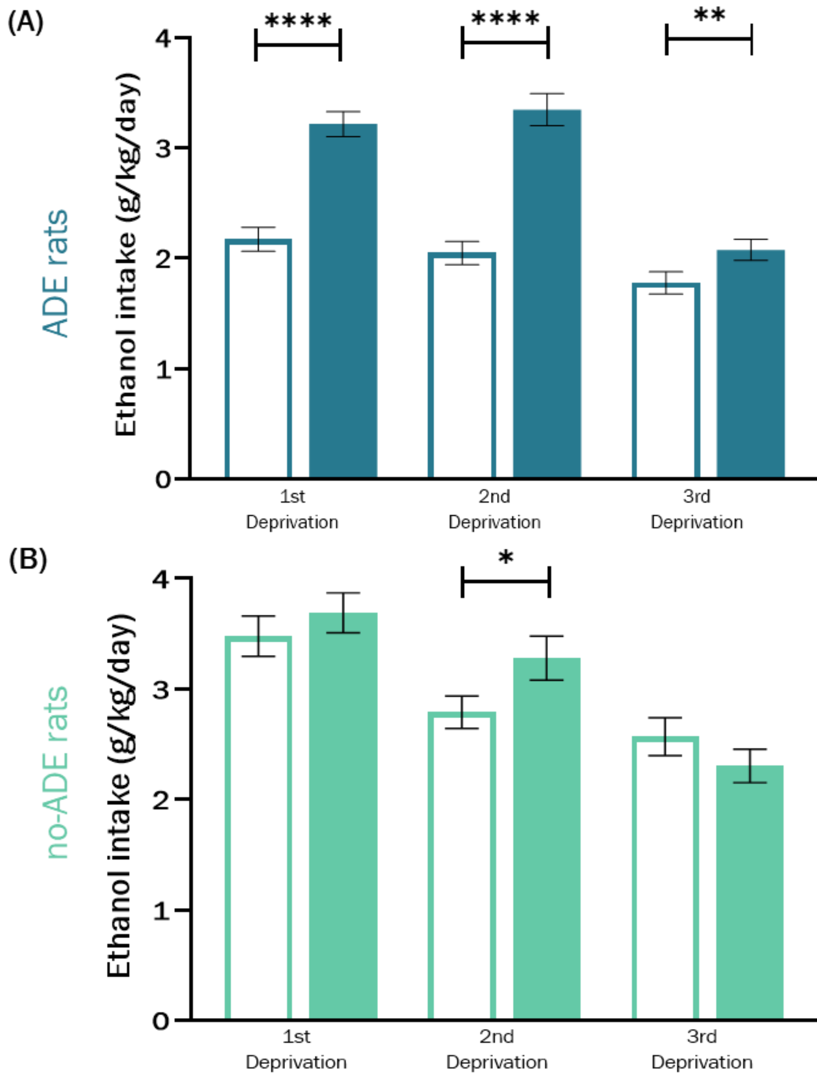


Figure 21. ***Ethanol intake*** *(expressed in g/kg/day) displayed by a cohort of 43 male rats. Bars represent the collapsed values of ethanol intake determined during the 3 days before (framed bars) and after (solid bars) each deprivation period, for* ***ADE rats*** *(n=30) (A) and* ***no-ADE rats*** *(n=13) (B). Data are represented as mean ± SEM. Statistical results, obtained through* ***a paired Student's t-tests*** *are represented [* p< 0.05; ** p<0.01; **** p<0.0001]*

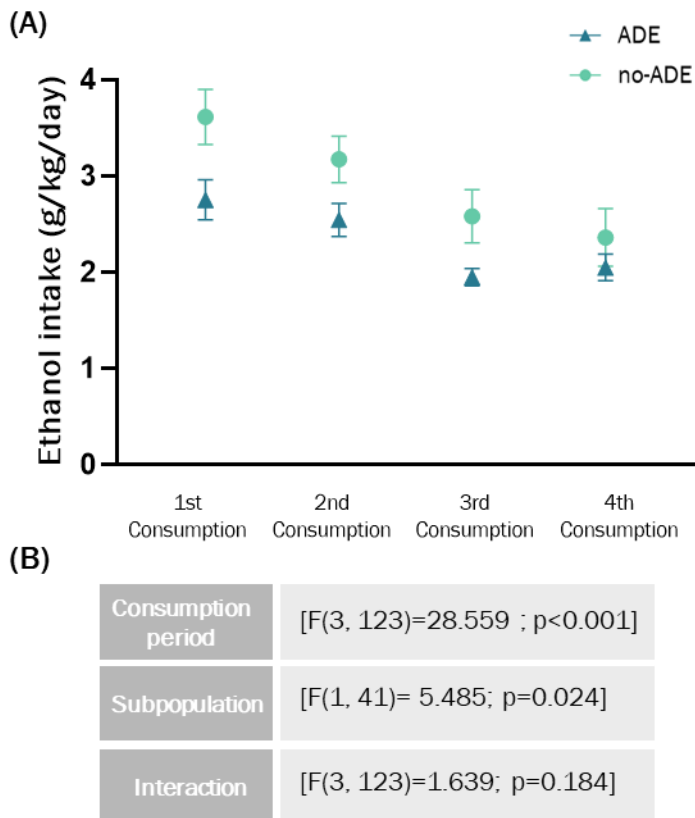
As shown in Figure 21, ADE and no-ADE rats seem to display different ethanol relapse-like drinking behaviour. Specifically:

- **ADE** animals (Figure 21 A) displayed a significant **increase in average ethanol intake** with respect to the basal value after abstinence, along the three consecutive deprivation periods assayed: [$t(29)=-7.521$; $p<0.0001$], [$t(29)=-5.046$; $p<0.0001$] , and [$t(29)=-2.605$; $p=0.014$], respectively, thus **confirming the ADE expression** in this group of male animals.

- Animals assigned to the **no-ADE** subgroup (Figure 21 B) clearly showed a different relapse-like drinking behaviour pattern. Concretely, neither in the first [$t(12)=-0.592$; $p=0.564$] nor in the third [$t(12)=1.391$; $p=0.189$] deprivation period did animals experience a significant increase in average ethanol intake. Although, in the second deprivation period an increase was detected, it was not very intense [$t(12)=-2.254$; $p=0.044$], confirming that these animals **did not show a clear trend to relapse-like drinking behaviour.**

Once animals were assigned to the ADE or no-ADE subpopulations, other experimental variables associated with ethanol consumption patterns, such as voluntary ethanol intake during the consumption period, total ethanol preference, or ethanol preferences for each ethanol solution (5, 10 and 20% v/v) were explored. Hence, we intended to analyse whether ADE and no-ADE groups displayed other phenotypic differences between them.

Collapsed voluntary ethanol consumption displayed by both groups along the four different consumption periods is plotted in Figure 22 A. Values from the 3 post-abstinence days in each period were excluded. These data were analysed using a mixed two-way ANOVA with consumption period being the within-group factor and subpopulation the between-group factor (Figure 22 B).



Consumption period	[F(3, 123)=28.559 ; p<0.001]
Subpopulation	[F(1, 41)= 5.485; p=0.024]
Interaction	[F(3, 123)=1.639; p=0.184]

*Figure 22. (A) Collapsed values of voluntary **ethanol intake** (expressed as g/kg/day) displayed by ADE (blue triangles, n=30) and no-ADE (green circles, n=13) at each **ethanol consumption period**. Values from the 3 post-abstinence days in each period are excluded. Data are represented as mean ± SEM. (B) Statistical results derived from mixed **two-way ANOVA** (consumption period x subpopulation) performed.*

As can be observed in Figure 22, the statistical analysis confirmed previous observations:

- The two-way ANOVA revealed the existence of significant differences between both subpopulations [$F(1,41)=5.484$; $p=0.024$] on ethanol intake.

- Moreover, the statistical analysis also detected a significant consumption period effect [$F(3,123)=28.559$; $p<0.001$] on voluntary ethanol consumption.

- However, no significant interaction effect was detected between both factors.

In order to further characterise both subpopulations, total ethanol preference was analysed. First of all, the average ethanol preference displayed by both subpopulations during the experiment 1.A is represented in Figure 23.

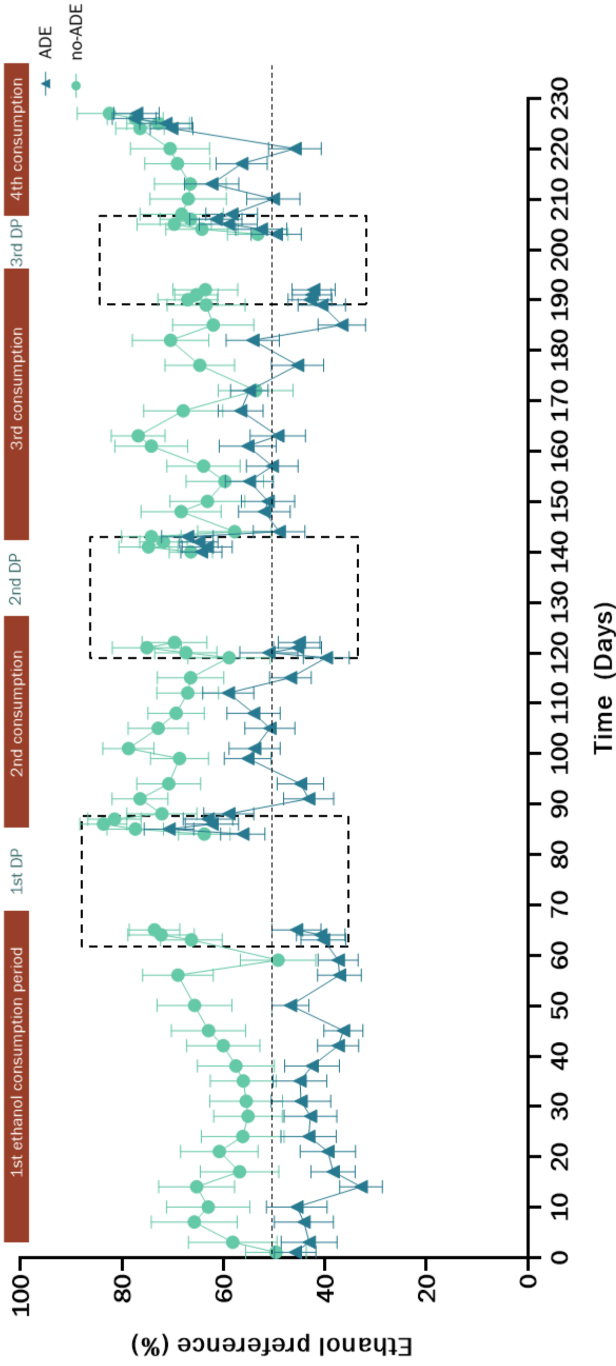
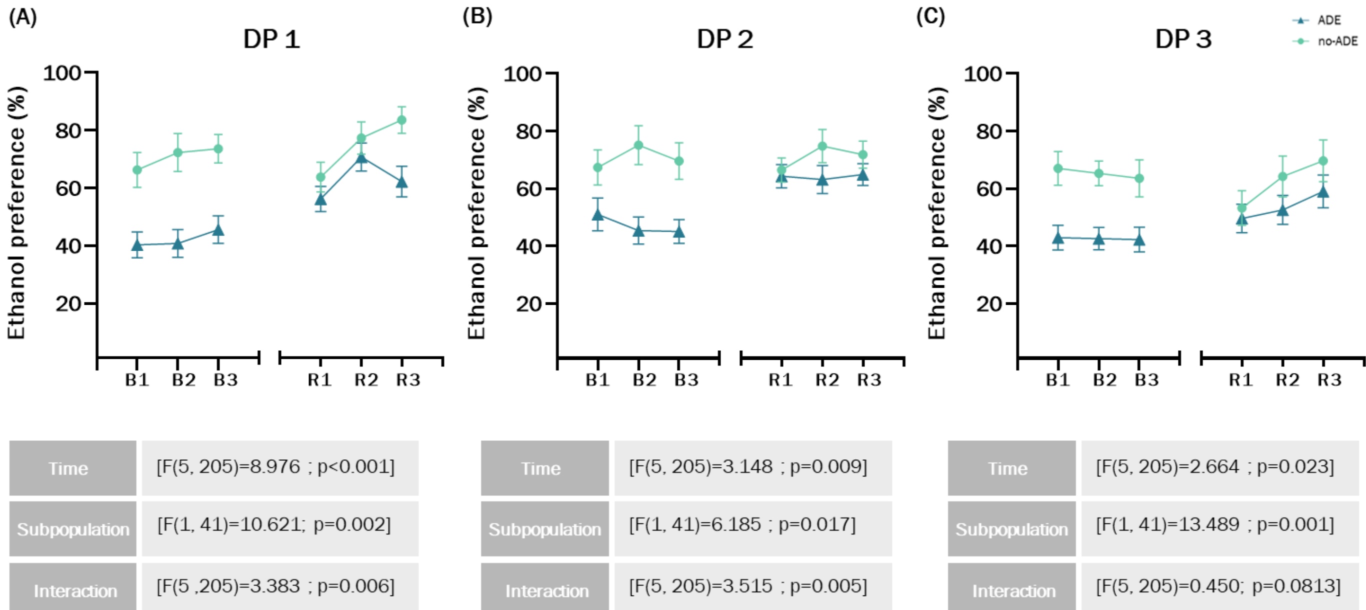


Figure 23. Average ***ethanol preference*** *(expressed in % of total fluid intake)* displayed by rats categorised in the **ADE** (blue triangles, n=30) or **no-ADE** (green circles, n=13) groups along the entire experiment. Dashed-line rectangles indicates the pre- and post-abstinence values at each period of deprivation. Data are represented as mean ± SEM. DP: deprivation period.

Figure 23 shows that, in a general sense, the **ADE group displayed lower ethanol preference than the no-ADE group.** Further analyses were performed to confirm this affirmation. First of all, we analysed if ethanol preference varied before and after abstinence in both groups. Hence, data included in each dashed-line rectangle were used to perform a mixed two-way ANOVA with the time being the within-groups factor and subpopulation the between-groups factor. This analysis was performed at each deprivation period. Ethanol preference along the three days before and after each deprivation period displayed are depicted in Figure 24. The statistical results obtained through the mixed two-Way ANOVA are also summarised at the bottom of Figure 24.



(A)	
Time	[F(5, 205)=8.976 ; p<0.001]
Subpopulation	[F(1, 41)=10.621; p=0.002]
Interaction	[F(5 ,205)=3.383 ; p=0.006]

(B)	
Time	[F(5, 205)=3.148 ; p=0.009]
Subpopulation	[F(1, 41)=6.185 ; p=0.017]
Interaction	[F(5, 205)=3.515 ; p=0.005]

(C)	
Time	[F(5, 205)=2.664 ; p=0.023]
Subpopulation	[F(1, 41)=13.489 ; p=0.001]
Interaction	[F(5, 205)=0.450; p=0.0813]

Figure 24. On the top: ***Ethanol preference*** *(expressed in % of total fluid intake) displayed by ADE (blue triangles, n=30) and no-ADE (green circles, n=13) animals during the days* ***before*** *(B1, B2 and B3)* ***and after*** *(R1, R2 and R3)* ***each deprivation period****: panel (A) includes data derived from the 1st deprivation period (DP1), panel (B) includes data derived from the 2nd deprivation period (DP2) and panel (C) includes data derived from the 3rd deprivation period (DP3).* Data are represented as mean ± SEM. DP: deprivation period. On the bottom: Statistical results from **two-way ANOVA** analysis of the depicted data (time x subpopulation).

As can be observed in Figure 24, the results of two-way ANOVA revealed the existence of **significant time and subpopulation effects, but also an interaction** between both factors in nearly all the deprivation periods assayed. To facilitate the interpretation of the obtained results, an additional statistical analysis was performed. Specificallly, data of the three days before and after each deprivation period was collapsed and compared through a paired Student's t-test (Figure 25) for both subpopulations.

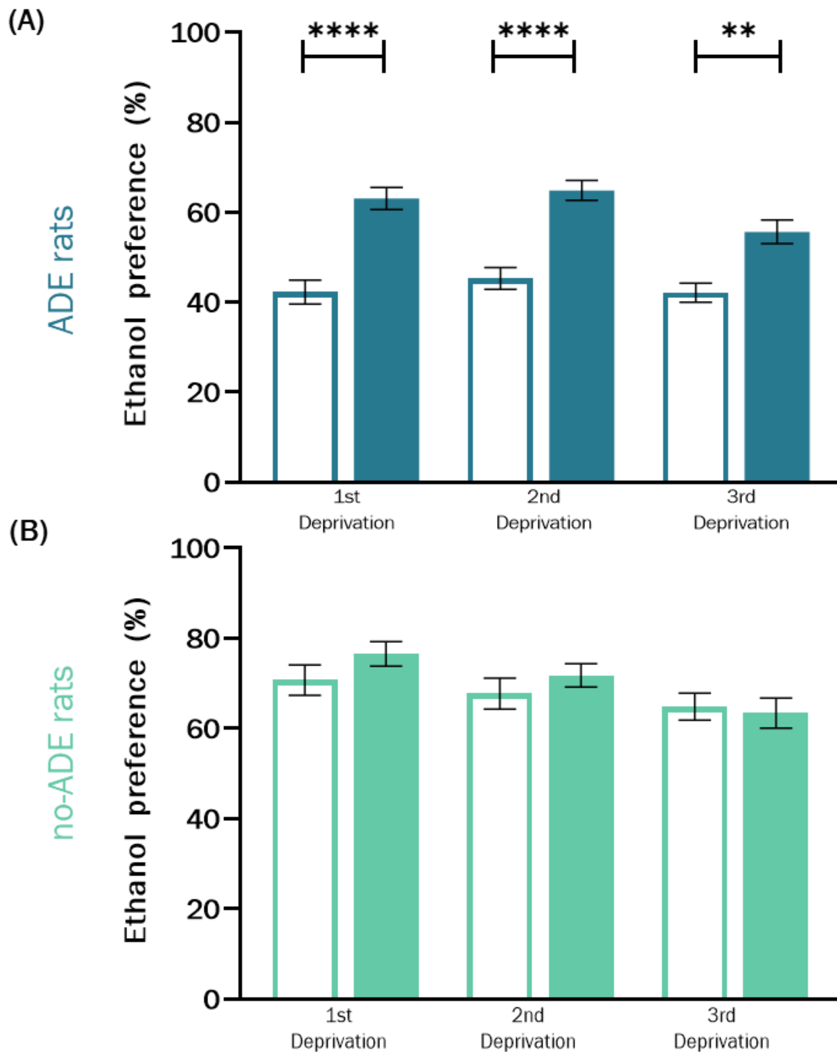


*Figure 25. **Ethanol preference** (expressed as % of total fluid intake) displayed by a cohort of 43 male rats. Bars represent the collapsed values of ethanol intake determined during the 3 days before (framed bars) and after (solid bars) each deprivation period, for **ADE rats** (n=30) (A) and **no-ADE rats** (n=13) (B). Data are represented as mean ± SEM. Statistical results, obtained through a **paired Student's t-tests** are represented [** p<0.01; **** p<0.0001]*

As depicted in Figure 25, when ethanol preference was compared between the ADE and no-ADE subgroups the results were even clearer than previously. The results of paired Student's t-test found that:

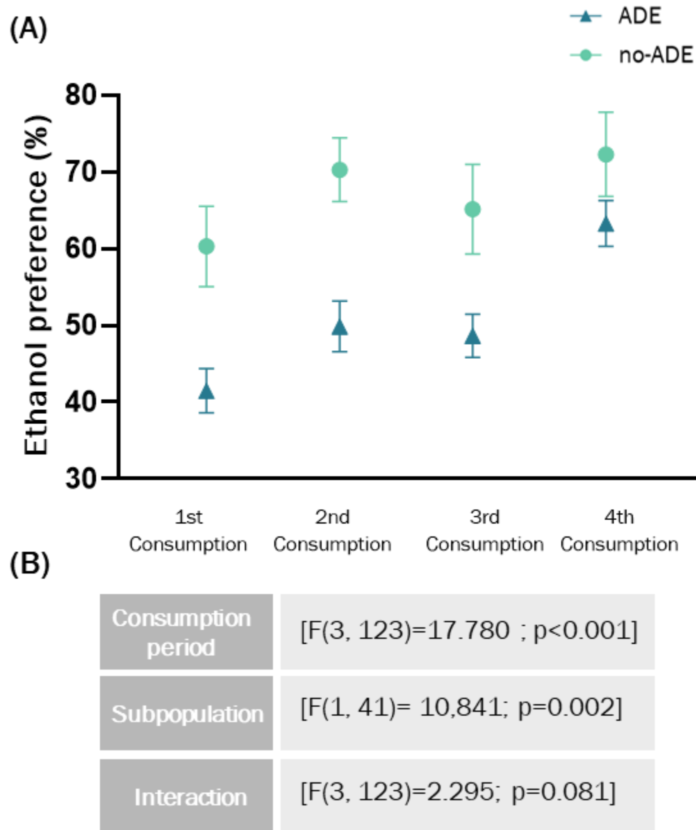
- Average ethanol preference significantly increased in the **ADE** group **from approximately 40% up to nearly 60%**, with respect to the basal value, along the three consecutive deprivation periods assayed: [$t(29)=-7.596$; $p<0.0001$], [$t(29)=-5.407$; $p<0.0001$], and [$t(29)=-3.084$; $p=0.004$], thus re-confirm the manifestation of the ADE phenomenon in this subpopulation of rats (Figure 25 A).

- Contrarily, animals assigned to the **no-ADE** subgroup (Figure 25 B) **did not show significant changes** in their ethanol preference after experiencing a deprivation period. Statistical analysis results were: [$t(12)=-1.259$; $p=0.231$] for the first deprivation period, [$t(12)=-0.054$; $p=0.957$] for the second one and [$t(12)=1.064$; $p=0.308$] for the third deprivation period.

Next, the average ethanol preference determined at each consumption period and along the time course of the experiment was analysed and compared between both subpopulations. For this purpose, a mixed two-way ANOVA with consumption period being the within-group factor and subpopulation the between-group factor was performed.

Figure 26 shows the average ethanol preference displayed by ADE and no-ADE rats along the four ethanol consumption periods assayed. Values from the 3 post-abstinence days in each period were excluded. Data were analysed using a mixed two-way ANOVA with consumption

period being the within-group factor and subpopulation the between-group factor



Consumption period	[F(3, 123)=17.780 ; p<0.001]
Subpopulation	[F(1, 41)= 10,841; p=0.002]
Interaction	[F(3, 123)=2.295; p=0.081]

Figure 26. (A) Collapsed values of ***ethanol preference*** *(expressed as % of total fluid intake) displayed by ADE (blue triangles, n=30) and no-ADE (green circles, n=13) at each* ***ethanol consumption period****. Values from the 3 post-abstinence days in each period are excluded. Data are represented as mean ± SEM. (B) Statistical results derived from mixed* ***two-way ANOVA*** *(consumption period x subpopulation) performed.*

As consigned in Figure 26 B, the statistical analysis determined:

- The existence of a significant consumption period [$F(3,123)=17.780$; $p<0.001$] and subpopulation [$F(1,41)=10.841$; $p=0.002$] effects. These results confirmed that, on one hand, **ADE and no-ADE subpopulations** displayed different ethanol preference and, on the other hand, that ethanol preference changed along the time of the experiment.

Particular preferences for each dilution (5%, 10% and 20% v/v) were also explored in both subpopulations. Experimental data are plotted in Figure 27. Firstly, these data were analysed through a three-way ANOVA with months being the within-group factor and subpopulation and ethanol dilution the between-group factors. The statistical results are summarised in Table 11.

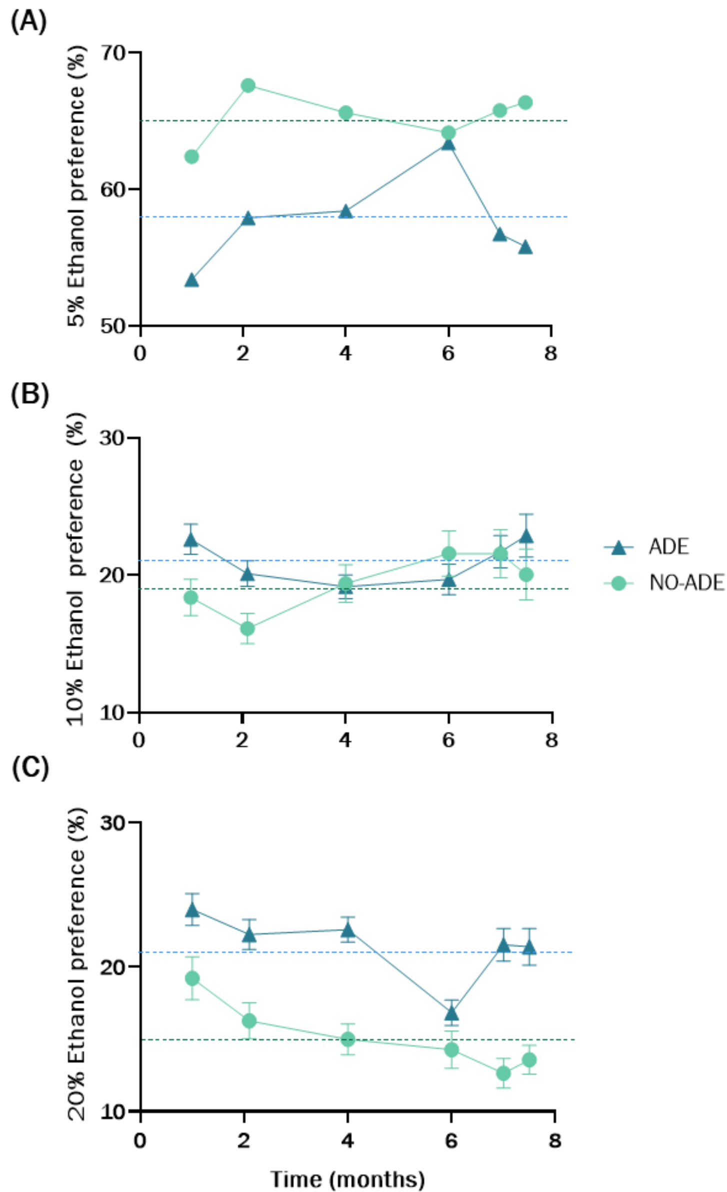


Figure 27. ***5*** *(A),* ***10*** *(B) and* ***20%*** *(C)* ***ethanol preference****, (expressed in % of each solution with respect to the total volume of alcohol consumed), displayed by* ***ADE*** *(blue triangles, n=30) and* ***no-ADE*** *(green circles, n=13) rats. Dashed lines represent average value for each group preference. Data are represented as mean ± SEM.*

Factor	Result
Months	$F_{6, 615}$=0.001; p=1.000
Ethanol dilution	$F_{2, 123}$=185,711; **p<0.001**
Subpopulation	$F_{1, 123}$<0.001; p=0.989
Months x Ethanol dilution	$F_{10, 615}$=1.831; p=0.052
Months x Subpopulation	$F_{5, 615}$=0.001; p=1.000
Ethanol dilution x Subpopulation	$F_{2, 123}$=3.944; **p=0.022**
Months x Ethanol dilution x Subpopulation	$F_{10, 615}$=1.103; p=0.357

Table 11. Results of ***three-way ANOVA*** *analysis (months x ethanol dilution x subpopulation) of* ***particular ethanol preference*** *on male rats.*

As can be observed in Figure 27, ADE and no-ADE rats displayed, once again, different behaviours that were confirmed by the statistical differences obtained with the three-way ANOVA analysis (Table 11). This analysis confirmed a significant effect of ethanol dilution (p<0.001) and a significant effect of ethanol dilution x subpopulation interaction (p=0.022) on particular ethanol preferences.

As the three-way ANOVA for repeated measures did not reveal a significant month effect, an additional 2-way ANOVA with consumption period being the within-group factors and subpopulation the between-

group factor, was performed. Collapsed experimental data are plotted in Figure 28.

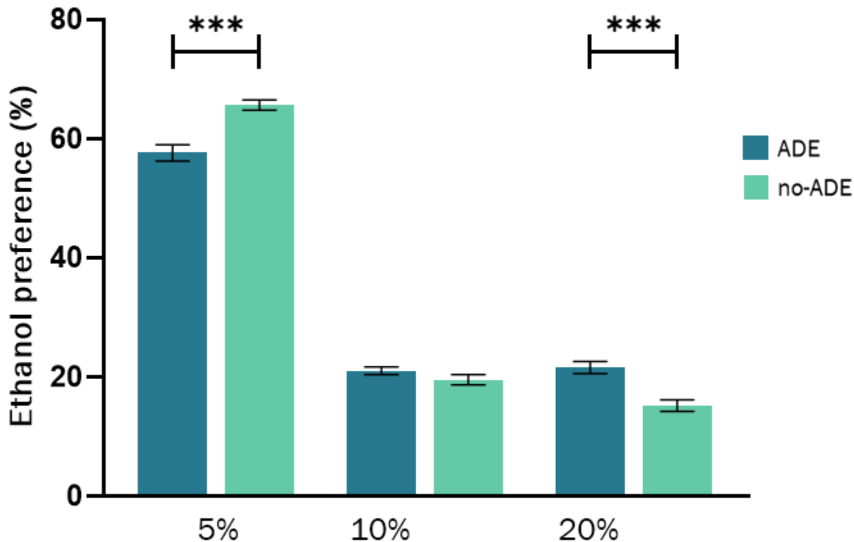


Figure 28. Collapsed data of particular ***ethanol preference 5%*** *(A),* ***10%*** *(B) and* ***20%*** *(C) (expressed in % of each solution with respect to the total volume of alcohol consumed) displayed by* ***ADE*** *(blue bars) and* ***no-ADE*** *(green bars) subpopulations. Data are represented as mean ± SEM. [* ** p < 0.001].*

When analysing particular ethanol preferences through a two-way ANOVA, animals, depending on the subpopulation considered, displayed a different ethanol consumption pattern. The analysis found a significant effect of ethanol dilution ($F_{2,30} = 1291.77$; $p < 0.001$) and, very interestingly, a significant interaction between subpopulation x ethanol dilution ($F_{2,30} = 27.75$; $p < 0.001$). The *post hoc* adjusted Bonferroni test determined that:

- The average preference for 5% ethanol dilution displayed by ADE and no-ADE rats was 57.86 ± 0.62% and 65.36 ± 0.83%, respectively, being statistically different ([$p<0.001$]. Hence, this result confirmed that **no-ADE rats showed a higher preference for 5% ethanol solution than ADE subpopulation.**

- On the other hand, the average preference for 20% ethanol dilution between ADE and no-ADE groups significantly differed ($p<0.001$). Concretely, **ADE** rats displayed a significantly **higher preference for 20% ethanol dilution** than no-ADE rats (21.46 ± 0.42% vs. 15.27 ±0.50%).

Finally, the total fluid intake and rat weight were registered and compared between both groups. The evolution of both parameters along the time course of the experiment is depicted in Figure 29.

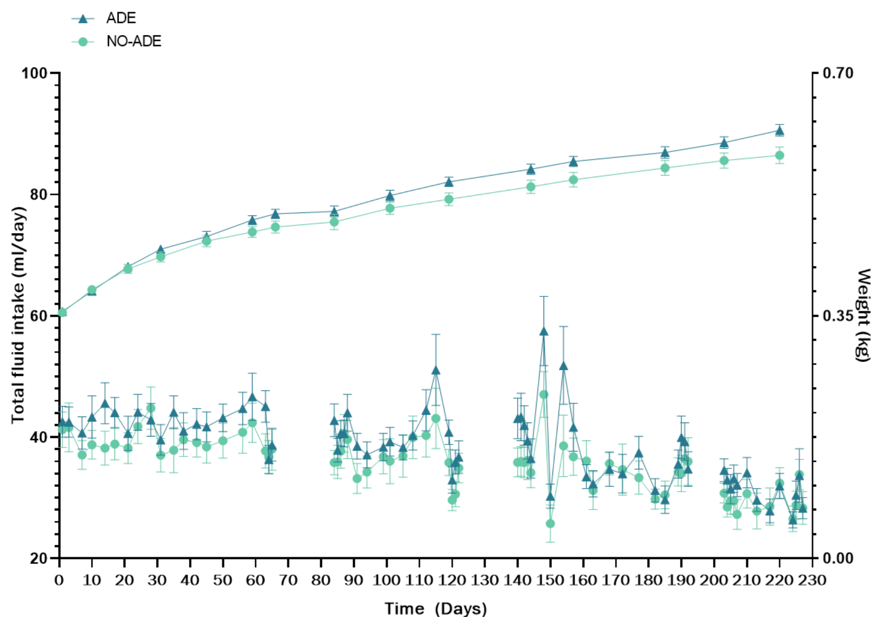


Figure 29. Average fluid intake and weight of ADE (blue triangles, n=30) and no-ADE (green circles, n=13) subpopulations. The Y axis in the left references the ***total fluid intake*** *(expressed in ml per day) during 32 weeks. The Y axis on the right registers the* ***weight*** *of the animals (expressed in kg) along the experiment. Data are represented as mean ± SEM.*

As can be observed and confirmed by the multiple t-test analysis, no differences between the two experimental groups were found neither for total fluid intake nor weight. ADE and no-ADE rats consumed an average volume of 38.62 ± 0.71 and 35.56 ± 0.55 ml/day respectively and the statistical analysis did not detect significant differences between them. Besides, their **growth-curves had a similar profile.** In fact, at the end of the experiment, ADE and no-ADE **rats showed similar weight,** being 612 ± 9 and 582 ± 12 g, respectively.

5.1.1.2 Experiment 1.2. Female relapse-like drinking behaviour determined under the ADE protocol

38 female rats were subjected to three deprivation periods under the ADE protocol for 26 weeks. After applying the above-mentioned criteria to categorise animals in the ADE or no-ADE group, 22 animals were assigned to the **ADE** group, whereas 16 rats were classified as **no-ADE** animals.

Figure 30 shows the average ethanol intake displayed, under the course of the experiment, by both experimental groups.

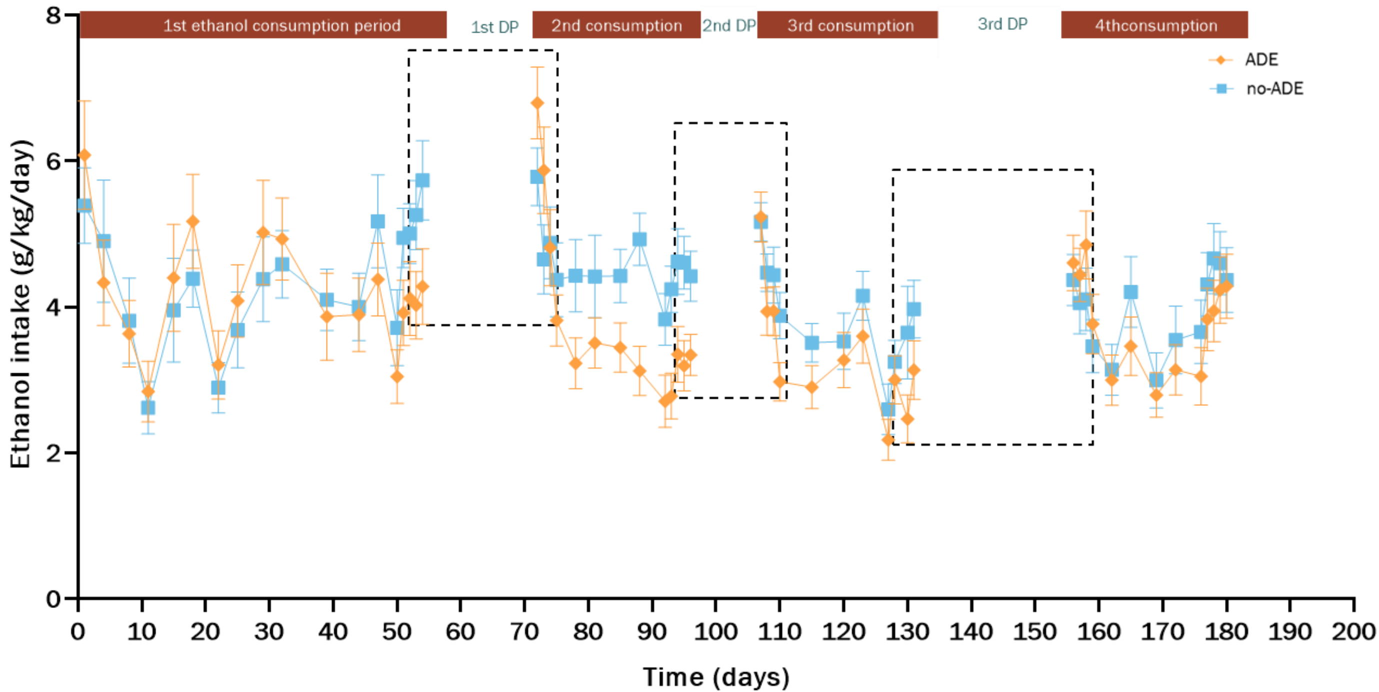
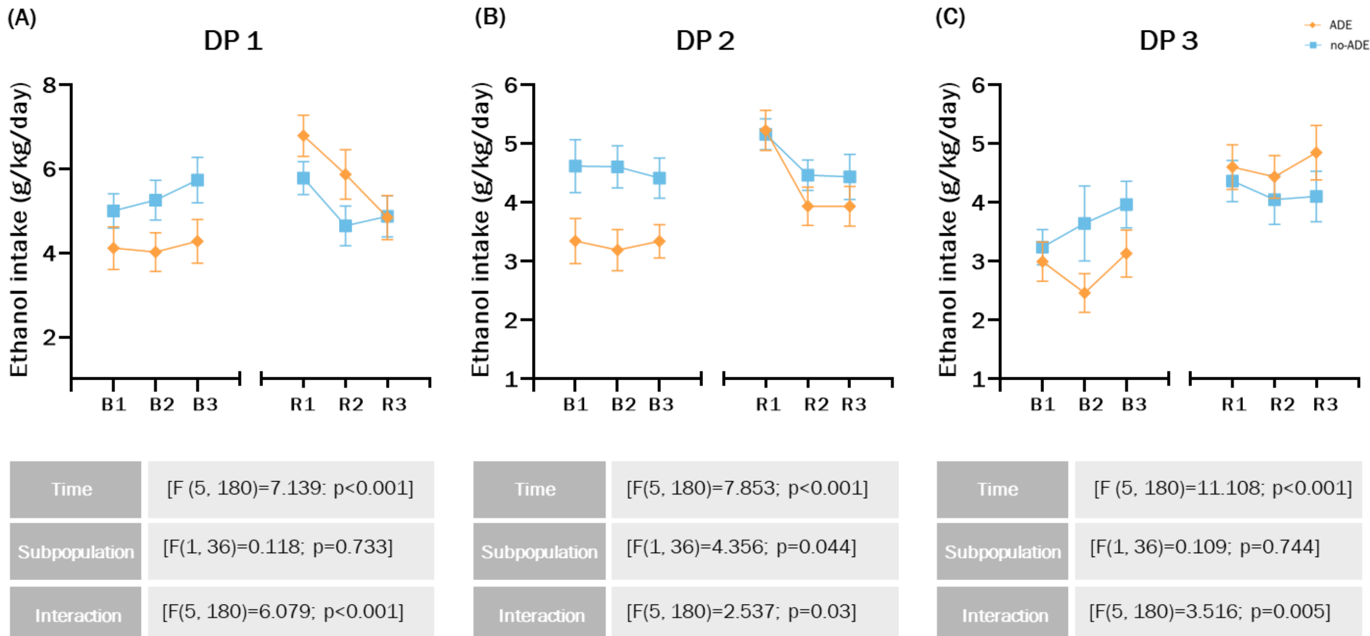


*Figure 30. Average voluntary **ethanol intake** (expressed as g/kg/day) displayed by rats categorised in the female ADE (orange rhombuses, n=22) or no-ADE (blue squares, n=16) groups along the entire experiment. Dashed-line rectangles indicate the pre-and post-abstinence values at each deprivation period. Data are represented as mean ± SEM. DP: deprivation period.*

As shown in Figure 30, at first sight, the voluntary ethanol intake displayed by our female cohort of rats along the time course of the experiment seemed to be **different between the two subpopulations** previously defined. Apparently, on some days, ADE rats displayed lower ethanol consumption than no-ADE animals. Data included in each dashed-line rectangle were used to perform a two-way ANOVA for repeated measures with time being the within-group factor and subpopulation the between-group factor. This analysis was performed at each deprivation period i.e., ethanol intake along the three days before and after each deprivation period displayed by both subpopulations (ADE and no-ADE) was compared.

The mean ethanol intake values included in this analysis and the statistical results obtained through the two-way ANOVA are plotted in Figure 31.



(A) DP 1	
Time	[F (5, 180)=7.139: p<0.001]
Subpopulation	[F(1, 36)=0.118; p=0.733]
Interaction	[F(5, 180)=6.079; p<0.001]

(B) DP 2	
Time	[F(5, 180)=7.853; p<0.001]
Subpopulation	[F(1, 36)=4.356; p=0.044]
Interaction	[F(5, 180)=2.537; p=0.03]

(C) DP 3	
Time	[F (5, 180)=11.108; p<0.001]
Subpopulation	[F(1, 36)=0.109; p=0.744]
Interaction	[F(5, 180)=3.516; p=0.005]

Figure 31. On the top: ***Ethanol intake*** *(expressed in g/kg/day) displayed by ADE (orange rhombuses, n=22) and no-ADE (blue squares, n=16) female animals during the days* ***before*** *(B1, B2 and B3) and* ***after*** *(R1, R2 and R3)* ***each deprivation period****: panel (A) includes data derived from the 1st deprivation period (DP1), panel (B) includes data derived from the 2nd deprivation period (DP2) and panel (C) includes data derived from the 3rd deprivation period (DP3). Data are represented as mean ± SEM.* DP: deprivation period. *On the bottom: Statistical results from* ***two-way ANOVA*** *analysis of the depicted data (time x subpopulation).*

As consigned in Figure 31, statistical analysis identified the existence of a **significant time effect** in the three deprivation periods analysed i.e., the rats manifested the ADE phenomenon. Very interestingly, a **significant interaction effect** between both factors (time and subpopulation) was detected in all situations. Considering these results, a paired Student's t-test collapsing data before and after each deprivation period was performed in order to confirm if animals displayed a relapse-like drinking behaviour depending on the subpopulation considered.

Figure 32 depicts the average ethanol intake of the three pre-abstinence and post-abstinence days for each deprivation period.

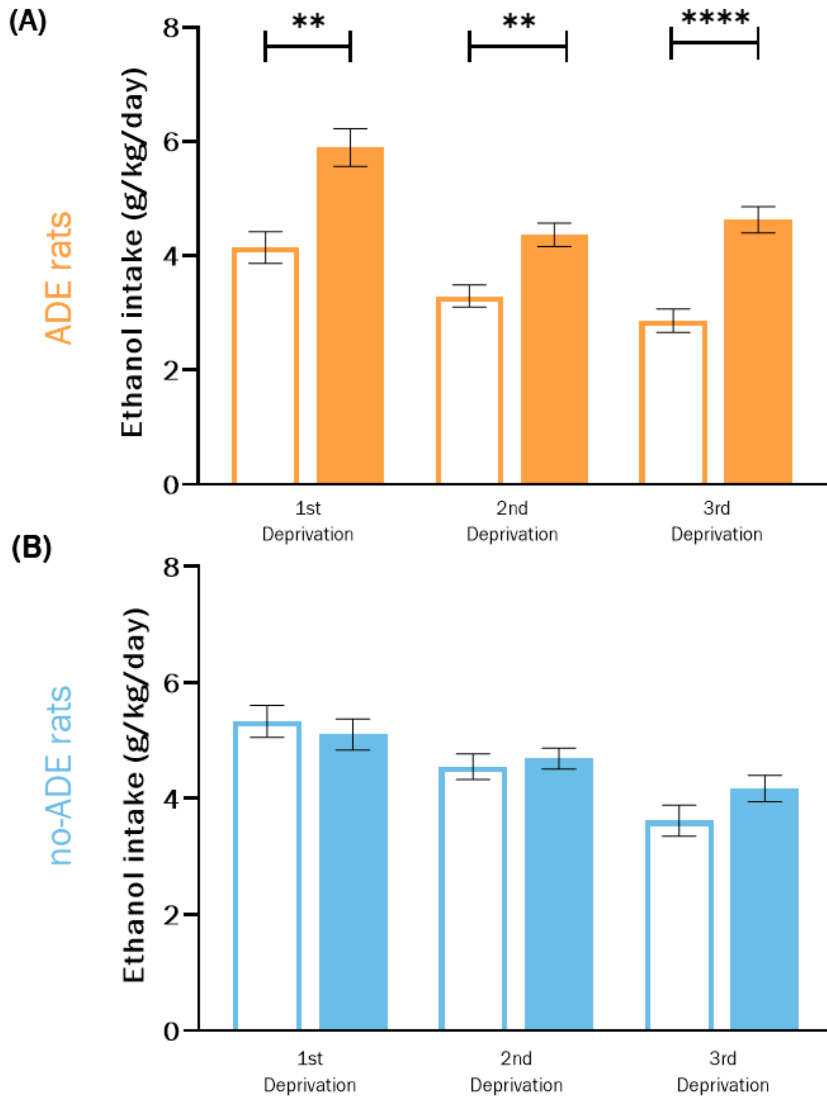


Figure 32. ***Ethanol intake*** *(expressed in g/kg/day) displayed by a cohort of 38 female rats. Bars represent the collapsed values of ethanol intake determined during the 3 days before (framed bars) and after (solid bars) each deprivation period, for* ***ADE rats*** *(n=22) (A) and* ***no-ADE rats*** *(n=16) (B). Data are represented as mean ± SEM. Statistical results, obtained through a* ***paired Student's t-tests*** *are represented [* p< 0.05; ** p<0.01; **** p<0.0001]*

As represented in Figure 32, paired t-test found that:

- **ADE female rats displayed a significant increase in ethanol intake** when compared with basal values (Figure 32 A) in the three deprivation periods assayed. The obtained statistical results were: [$t(21)=3.415$; $p=0.003$], [$t(21)=3.332$; $p=0.003$] and [$t(21)=5.161$; $p<0.0001$], respectively. These results confirmed the manifestation of the relapse-like drinking behaviour in this subpopulation of rats.

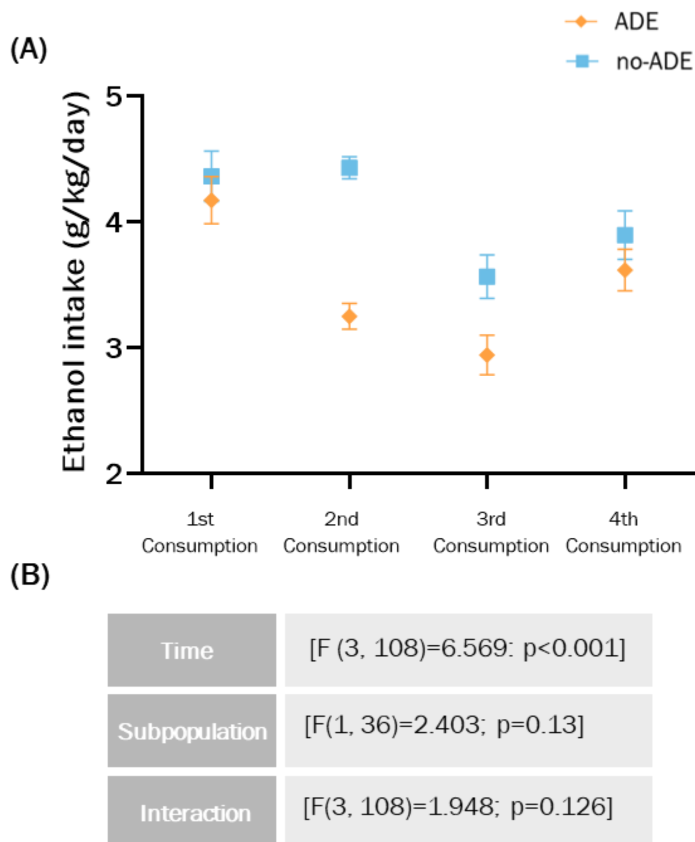
- On the contrary, female rats assigned to the **no-ADE group did not display the ADE phenomenon** (Figure 32 B). The statistical results in the three deprivation periods were: [$t(15)=0.758$; $p=0.46$], [$t(15)=0.514$; $p=0.615$], and [$t(15)=1.283$; $p=0.219$].

As previously shown for male rats, after the classification of each female rat to one of the two subpopulations depending on their relapse-like drinking behaviour, other experimental variables associated with ethanol consumption pattern, such as voluntary ethanol intake during the consumption period, total ethanol preference, or ethanol preferences for each ethanol solution (5, 10 and 20% v/v) were explored so as to find out whether ADE and no-ADE groups displayed other phenotypic differences between them.

First of all, the comparison of the average voluntary ethanol intake between ADE and no-ADE female animals along the four ethanol consumption periods was performed through a mixed two-way ANOVA with <u>time</u> being the within-group factor and <u>subpopulation</u> the between-

group factor. The values from the 3 post-abstinence days in each period were excluded.

Figure 33 plots the comparison of average voluntary ethanol intake displayed by ADE and no-ADE female rats along the four ethanol consumption periods assayed as well as the statistical results obtained after performing the two-way ANOVA test.



Time	[F (3, 108)=6.569; p<0.001]
Subpopulation	[F(1, 36)=2.403; p=0.13]
Interaction	[F(3, 108)=1.948; p=0.126]

*Figure 33. Collapsed values of voluntary **ethanol intake** (expressed as g/kg/day) displayed by female ADE (orange rhombuses, n=22) and no-ADE (blue squares, n=16) at each **ethanol consumption period**. Values from the 3 post-abstinence days in each period are excluded. Data are represented as mean ± SEM. (B) Statistical results derived from mixed **two-way ANOVA** (consumption period x subpopulation) performed.*

The statistical analysis revealed that **ethanol intake significantly varied depending on time** [$F(3,108)=6.569$: $p<0.001$], but no significant differences were found between both subpopulations [$F(1,36)=2.403$; $p=0.13$]. Furthermore, the interaction between both factors (time and subpopulation) was not significant [$F(3,108)=1.948$; $p=0.126$].

In order to deepen the characterisation of both subpopulations, ethanol preference was analysed in both groups. To begin with, the average ethanol preference displayed by each subpopulation (ADE and no-ADE) during the course of experiment 1.B is depicted in Figure 34.

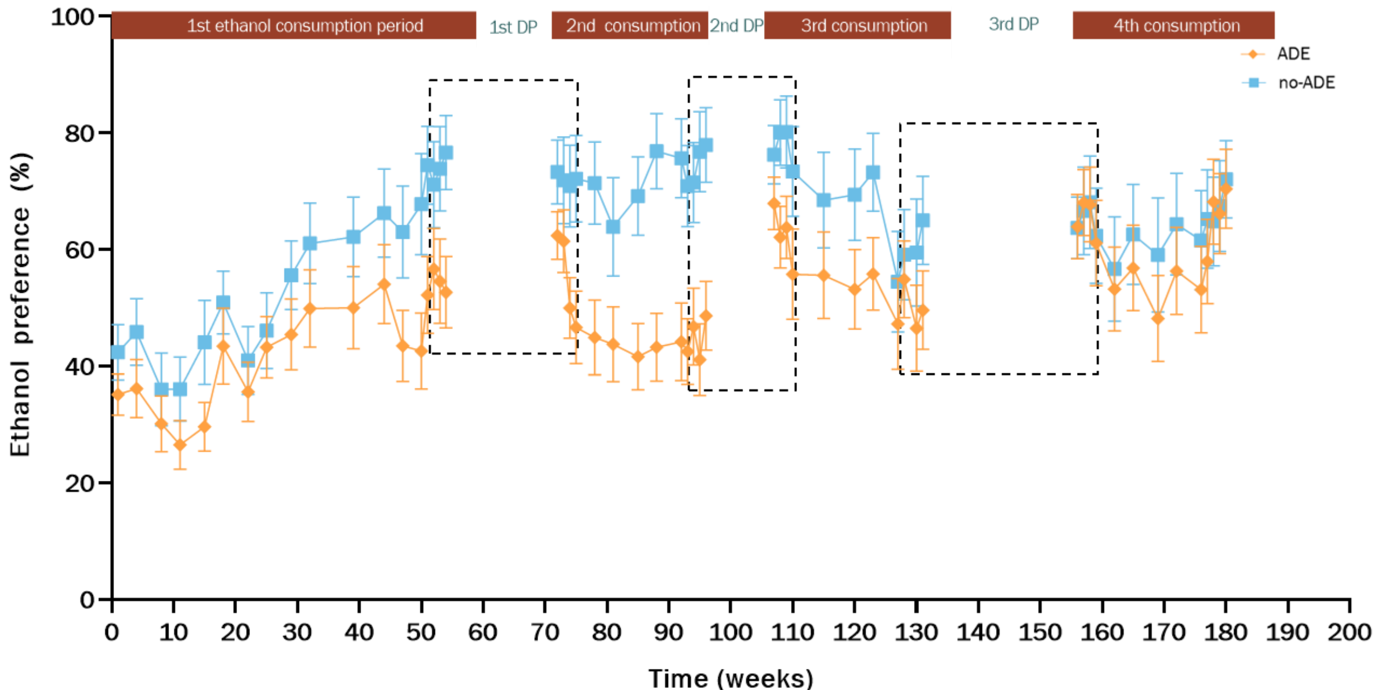
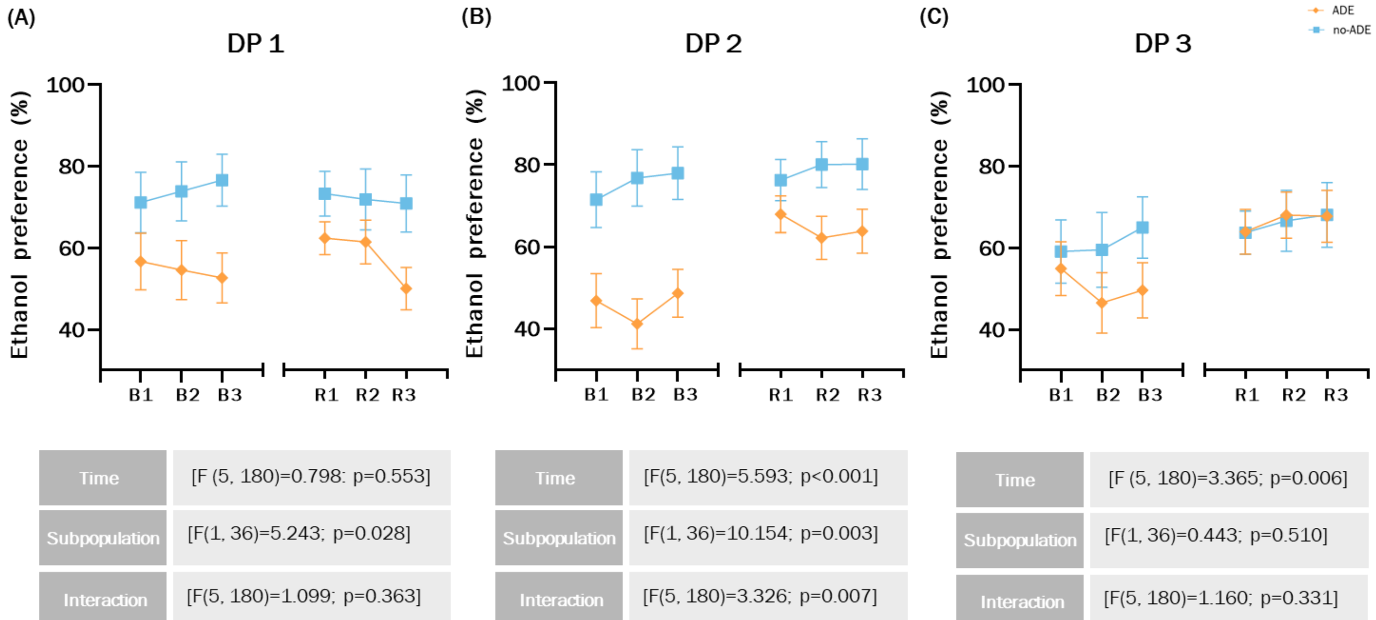


Figure 34. Average ***ethanol preference*** *(expressed in % of total fluid intake)* displayed by female rats categorised in the **ADE** (orange rhombuses, n=22) or **no-ADE** (blue squares n=16) groups along the entire experiment. Dashed-line rectangles indicates the pre- and post-abstinence values at each period of deprivation. Data are represented as mean ± SEM. DP: deprivation period.

As depicted in Figure 34, **ADE females displayed lower ethanol preference than no-ADE group** along the time course of the experiment. Further analyses were performed to confirm it. Firstly, we analysed if ethanol preference varied after abstinence in both groups. To this end, data included in each dashed-line rectangle were used to perform a two-way ANOVA for repeated measures with time being the within-group factor and subpopulation the between-group factor. This analysis was performed at each deprivation period. That is to say, ethanol intake values along the three days before and after each deprivation period displayed by both subpopulations (ADE and no-ADE) were compared.

Figure 35 represents the ethanol preference values of the days included in this analysis and the obtained statistical results through the two-way ANOVA analysis.



(A)

Time	[F (5, 180)=0.798: p=0.553]
Subpopulation	[F(1, 36)=5.243; p=0.028]
Interaction	[F(5, 180)=1.099; p=0.363]

(B)

Time	[F(5, 180)=5.593; p<0.001]
Subpopulation	[F(1, 36)=10.154; p=0.003]
Interaction	[F(5, 180)=3.326; p=0.007]

(C)

Time	[F (5, 180)=3.365; p=0.006]
Subpopulation	[F(1, 36)=0.443; p=0.510]
Interaction	[F(5, 180)=1.160; p=0.331]

Figure 35. On the top: ***Ethanol preference*** *(expressed in % of total fluid intake) displayed by female ADE (orange rhombuses, n=22) and no-ADE (blue squares, n=16) animals during the days* ***before*** *(B1, B2 and B3)* ***and after*** *(R1, R2 and R3)* ***each deprivation period:*** *panel (A) includes data derived from the 1st deprivation period (DP1), panel (B) includes data derived from the 2nd deprivation period (DP2) and panel (C) includes data derived from the 3rd deprivation period (DP3).* Data are represented as mean ± SEM. DP: deprivation period. On the bottom: Statistical results from **two-way ANOVA** analysis of the depicted data (time x subpopulation).

As shown in Figure 35, when total ethanol preferences were analysed in the female subpopulations, the obtained results were not very consistent, i.e., they are different depending on the deprivation period:

- In the first deprivation period (Figure 35 A), only a subpopulation significant effect was detected [$F(1,36)=5.243$; $p=0.028$].

- In the second deprivation period (Figure 35 B), the statistical analysis identified not only a significant subpopulation effect [$F(1,36)=10.154$; $p=0.003$], but also a time effect [$F(5,180)=5.593$; $p<0.001$] and an interaction effect [$F(5,180)=3.326$; $p=0.007$].

- However, in the third deprivation period (Figure 35 C), only a time effect was detected [$F(5,180)=3.365$; $p=0.006$].

Like the previous section, ethanol preference values before and after each deprivation period were collapsed and compared through a paired Student's t-test. Hence, Figure 36 depicts the average ethanol preference of the three pre-abstinence and post-abstinence days for each deprivation period in both subpopulations.

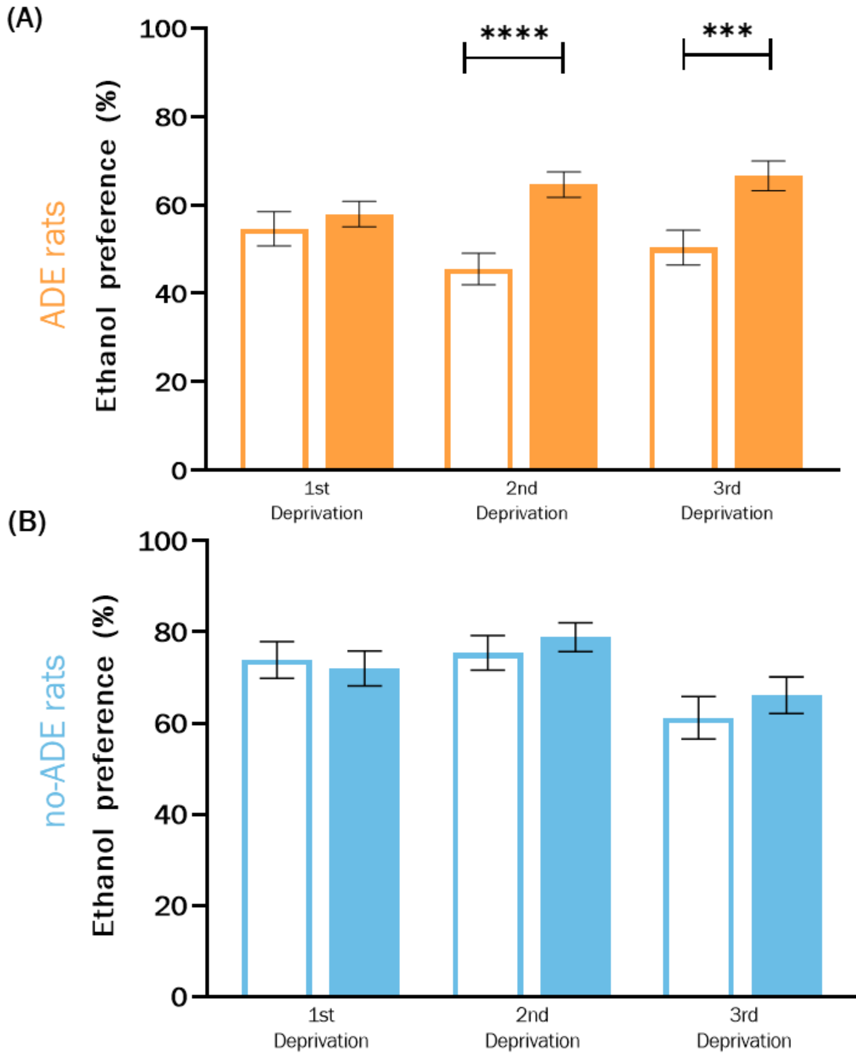


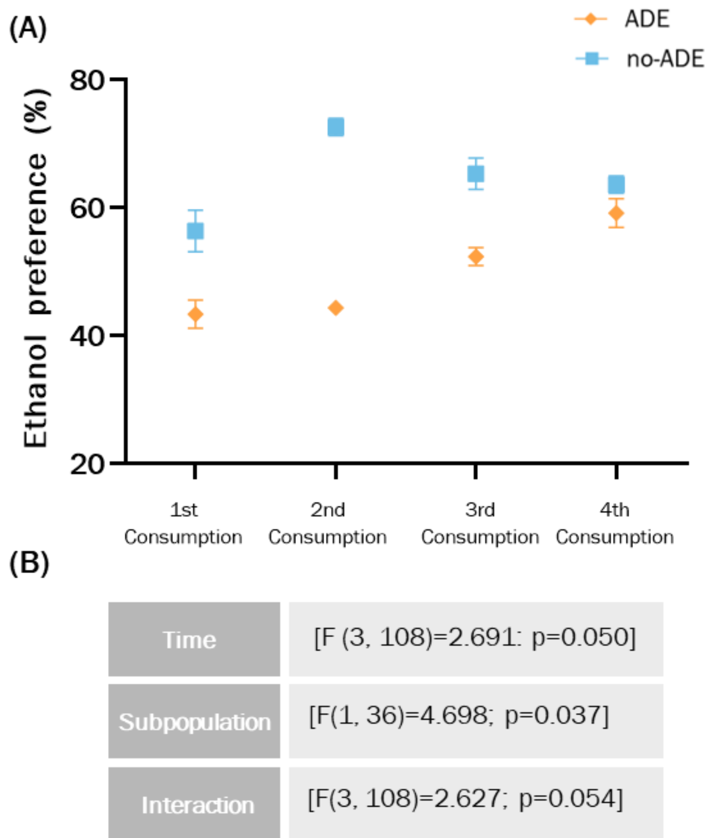
*Figure 36. **Ethanol preference** (expressed as % of total fluid intake) displayed by a cohort of 38 female rats. Bars represent the collapsed values of ethanol intake determined during the 3 days before (framed bars) and after (solid bars) each deprivation period, for **ADE rats** (n=22) (A) and **no-ADE rats** (n=16) (B). Data are represented as mean ± SEM. Statistical results, obtained through a **paired Student's t-tests** are represented [*** p<0.001; **** p<0.0001]*

The analysis of data represented in Figure 36 revealed that:

- **ADE female rats** did not did not increase their ethanol preference after the first deprivation period [$t(65)=0.846$; $p=0.401$]. Nonetheless, a significant increase in their preference for ethanol **after the second and the third deprivation period** with respect to their basal value was observed: [$t(65)=5.015$; $p<0.0001$] and [$t(65)=3.583$; $p<0.001$], respectively. These results suggest that the drinking behaviour of the ADE group was not constant during all the experiment.

- Conversely, the **no-ADE female group did not change their ethanol preference regardless of the deprivation period analysed.** Statistical analysis results were: [$t(47)=0.763$; $p=0.450$] for the first deprivation period, [$t(47)=1.387$; $p=0.172$ for the second one and [$t(47)=1.131$; $p=0.264$] for the third deprivation period.

Next, the ethanol preference displayed by both groups along the four consumption periods was also analysed. Values from the 3 post-abstinence days after each deprivation period were excluded. Concretely, a mixed two-way ANOVA with consumption period being the within-group factor and subpopulation the between-group factor was performed. The experimental data and the results obtained through the mixed two-way ANOVA analysis are consigned in Figure 37.



Time	[F (3, 108)=2.691; p=0.050]
Subpopulation	[F(1, 36)=4.698; p=0.037]
Interaction	[F(3, 108)=2.627; p=0.054]

Figure 37. (A) Collapsed values of ***ethanol preference*** *(expressed as % of total fluid intake) displayed by female ADE (orange rhombuses, n=22) and no-ADE (blue squares, n=16) rats at each* ***ethanol consumption period****. Values from the 3 post-abstinence days in each period are excluded. Data are represented as mean ± SEM. (B) Statistical results derived from mixed* ***two-way ANOVA*** *(consumption period x subpopulation) performed.*

According to Figure 37 A, the differences in ethanol preference displayed by female ADE and no-ADE rats seem to be dampened over time. Thus, the statistical analysis detected:

- A significant subpopulation effect [F(1,36)=4.698; p=0.037], meaning that the **ethanol preference displayed by female ADE and**

no-ADE animals is significantly different. Specifically, as can be observed, the ADE group displayed a lower ethanol preference.

- Besides, the time effect was also significant [$F(3,108)=2.691$; $p=0.050$], and the interaction between both factors was nearly significant [$F(3,108)=2.627$; $p=0.054$].

Finally, to conclude the characterisation of both groups with respect to their ethanol consumption pattern, particular preferences for each ethanol dilution (5%, 10% and 20% v/v) displayed by each subpopulation over time were also analysed. For this purpose, a three-way ANOVA (with months being the within-group factor and subpopulation and ethanol dilution the between-group factors) was performed. Experimental data are plotted in Figure 38 and the statistical results obtained through the three-way ANOVA test are summarised in Table 12.

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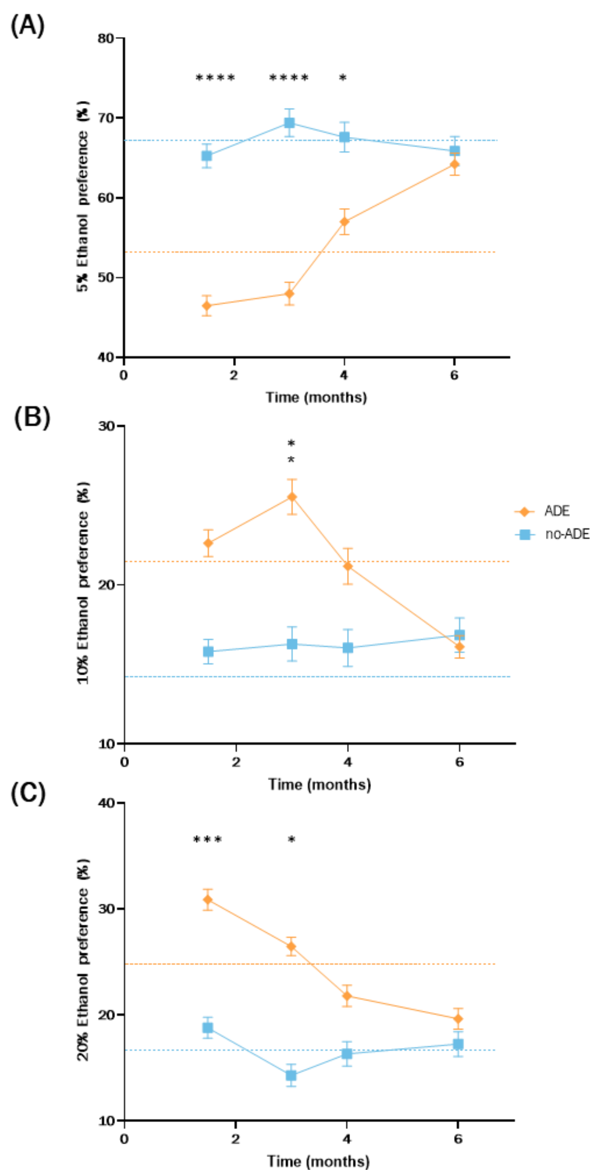


*Figure 38. **5** (A), **10** (B) and **20%** (C) **ethanol preference**, (expressed in % of each solution with respect of the total volume of alcohol consumed), displayed by female **ADE** (orange rhombuses, n=22) and **no-ADE** (blue squares, n=16) rats. Dashed lines represent average value for each group preference. Data are represented as mean ± SEM. Bonferroni post-hoc test was used to analyse data. [*p<0.05; *** p<0.001; **** p<0.0001]*

Factor	Result
Months	$F_{3, 324}<0.001$; $p=1.000$
Ethanol dilution	$F_{2, 108}=163.559$; **$p<0.001$**
Subpopulation	$F_{1, 108}<0.001$; $p=0.996$
Months x Ethanol dilution	$F_{6, 324}=5.049$; **$p<0.001$**
Months x Subpopulation	$F_{3, 324}<0.001$; $p=1.000$
Ethanol dilution x Subpopulation	$F_{2, 108}=9.636$; **$p<0.001$**
Months x Ethanol dilution x Subpopulation	$F_{6, 324}=5.527$; **$p<0.001$**

Table 12. Results of Three-Way ANOVA analysis (months x ethanol dilution x subpopulation) of ***particular ethanol preference*** *on female rats.*

As depicted in Figure 38, ADE and no-ADE rats displayed **different preferences for each ethanol dilution.** This observation was confirmed by the three-way ANOVA analysis (Table 12). A **significant effect of month x ethanol dilution x subpopulation interaction** ($p<0.001$) on particular ethanol preferences was detected. Figure 38 includes the significance of Bonferroni *post hoc* test performed to compare the ethanol preference displayed in each timepoint by each group:

- By and large, **ADE subpopulation** displayed a significantly higher preference for 10 and 20% ethanol dilutions while no-ADE rats displayed a significant higher preference for 5% ethanol dilution.

- However, **these differences tended to disappear towards the end of the experiment.** These results suggest that the differences on ethanol preference displayed by ADE and no-ADE female animals are not as consistent as those found among male rats' population.

Finally, the total fluid intake and rat weight were registered and compared between both groups of female rats. The evolution of both parameters during the time course of the experiment is represented in Figure 39.

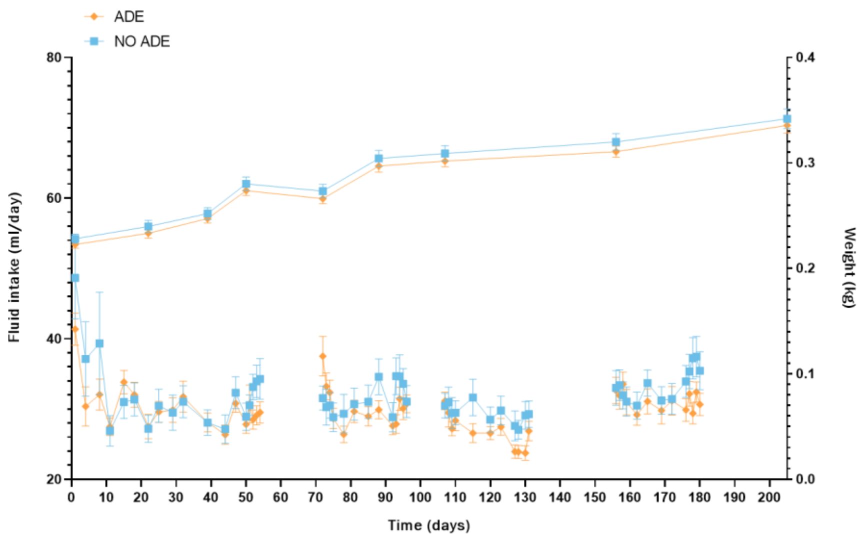


*Figure 39. Average fluid intake and weight of female ADE (orange rhombuses, n=22) and no-ADE (blue squares, n=16) subpopulations. The Y axis in the left references the **total fluid intake** (expressed in ml per day) during 26 weeks. The Y axis on the right registers the **weight** of the animals (expressed in kg) along the experiment. Data are represented as mean ± SEM.*

As can be observed in Figure 39, and confirmed by the multiple t-test analysis, **no differences between the two experimental groups were found neither for total fluid intake nor weight.** ADE and no-ADE female animals displayed an average fluid intake 29.84 ± 0.41 and 31.80 ± 0.49 ml/day, respectively. Their weight evolution was not different, at the end of the experiment, ADE and no-ADE rats' weight was similar, 336 ± 7 and 342 ± 9 g, respectively.

5.1.2 Experiment 2. Study of the Efficacy of NAC in the prevention of alcohol relapse-like drinking.

The ADE subpopulations identified in experiments 1.A and 1.B repeatedly displayed an ethanol relapse-like drinking behaviour. Consequently, they were considered adequate experimental subjects to carry out experiments 2.A and 2.B. These experiments were conducted in order to test the efficacy of NAC in the prevention of ADE phenomenon. To do so, animals experienced a new deprivation period that lasted 21 days, during which they were treated with NAC or vehicle during 14 days: 10 days in abstinence and 4 days after ethanol reintroduction. The comparison of the ethanol consumption displayed by rats before and after the fourth abstinence period was used to detect the manifestation, or not, of the ADE phenomenon.

5.1.2.1 Experiment 2.a. Preclinical study in male Wistar rats

The experiment 2.A was performed with 30 male Wistar rats categorised as ADE animals in experiment 1.A. During the fourth deprivation period, these animals were randomly distributed between three experimental groups (n=10 per group) treated with **vehicle, NAC 60 mg/kg or NAC 100 mg/kg.**

Figure 40 shows the daily time course (left panel) and the 4-day average intake of ethanol (right panel) before (basal) and after (post-abstinence) the deprivation period that lasted 21 days.

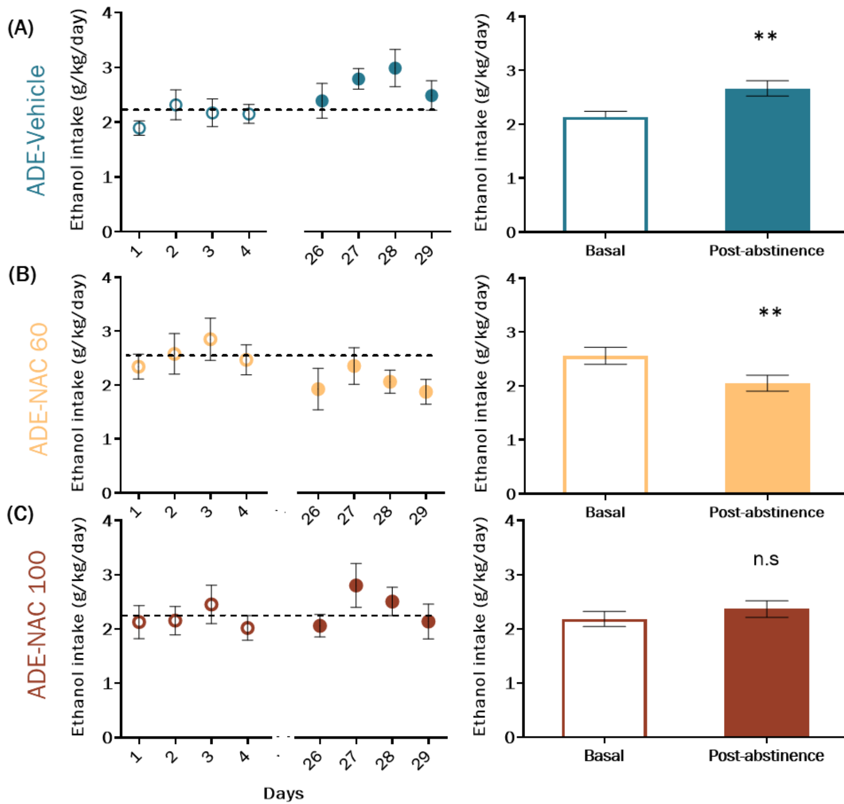


Figure 40. Effect of the subcutaneous injections of NAC on ADE male animals, once a day for 14 days of ***vehicle*** *(A),* ***NAC 60 mg/kg*** *(B) or* ***NAC 100 mg/kg*** *(C) on ethanol intake (n = 10/group). On the left part of A, B, and C panels, the mean daily ethanol intake determined during the 4 days of baseline and post-abstinence days is shown. Data were analysed by one-way ANOVA for repeated measures. The dashed line represents the mean baseline value. 1, 2, 3, and 4 represent the baseline measurements taken throughout 4 days prior to the abstinence period whereas 26, 27, 28 and 29 represent the post-abstinence measurements. On the right part of panels A, B, and C, the collapsed values of alcohol intake determined during the 4 days of baseline and the post-abstinence period are represented as bars; the results of the paired Student's t test for the ethanol intake are included. Data are represented as mean ± SEM. [** p<0.01; n.s. p>0.05]*

As can be observed in Figure 40, each group of animals displayed a different relapse-like drinking behaviour depending on the received treatment:

- After the abstinence period, **vehicle-treated animals showed a significant increase in ethanol intake** in comparison with that observed in the baseline period (Figure 40 A). On one hand, one-way ANOVA for repeated measures detected a statistically significant effect on daily alcohol intake over time [$F(7,63)= 2.362$; $p= 0.033$]. On the other hand, student's t test for paired measures determined that average ethanol intake increased significantly after the deprivation period up to 25%, from 2.14 to 2.67 g/kg/day [$t(39)=-2.960$; $p= 0.005$]. Thus, confirming that vehicle-treated rats **manifested the ADE phenomenon.**

- As can be appreciated in Figure 40 B and Figure 40 C, a 14-day treatment based on one daily subcutaneous **NAC injection of 60 or 100 mg/kg**, was able to **block the expression of the ADE phenomenon.** These results suggest the potential ethanol anti-relapse effect of NAC.

- In the case of animals treated with the **NAC 60 mg/kg dose** (Figure 40 B), a significant **decrease** (around −20%) **in ethanol intake** after the deprivation period (from 2.56 to 2.1 g/kg/day) was detected. The effect on daily ethanol intake over time was statistically significant, as confirmed by ANOVA for repeated measures [$F(7,63)= 2.155$; $p= 0.05$], and also the paired t test for collapsed values [$t(39)=3.110$; $p= 0.003$].

- When rats received the **NAC 100 mg/kg treatment** (Figure 40 C) statistical analysis did not reveal significant differences in ethanol intake between basal values (2.19 g/kg/day) and post-abstinence values (2.37 g/kg/day) since neither ANOVA for repeated measures detected a statistically significant effect on daily alcohol consumption over time [$F(7,63)=1.166$; $p=0.335$] nor did the Student's t test determine differences between mean ethanol intake values [$t(39)=-0.980$; $p= 0.333$].

The possible effect of NAC treatment on fluid or food consumption was evaluated. Hence, total fluid intake and animal weight before and after treatment were measured and compared. These data are collected in Figure 41.

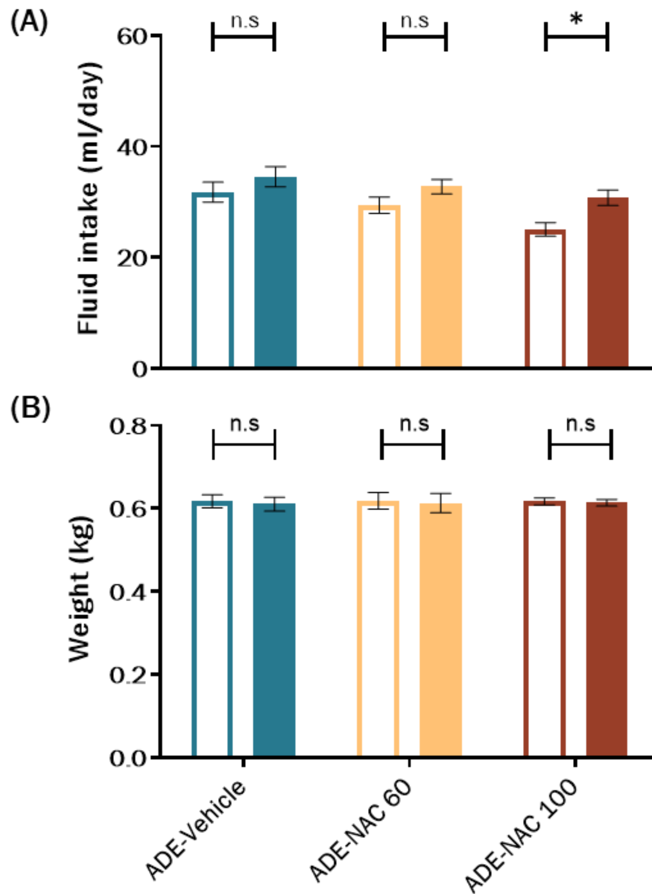


Figure 41. Effect of NAC treatment on ***fluid intake*** *(A) and* ***body weight*** *(B) of male Wistar rats. Fluid intake was determined during the four days before and after NAC administration whereas weight was determined once before and after NAC administration. Framed bars represent basal values whereas solid bars represent post-treatment values. Data are represented as mean ± SEM. Paired Student's t-test was applied at each experimental condition [* $p<0.05$; n.s. $p>0.05$]*

As can be seen in Figure 41 and confirmed by the statistical analysis, the total fluid intake (Figure 41 A) did not change during the post-

abstinence period after vehicle treatment [t(39)=−1.562; p=0.126] nor in the 60 mg/kg NAC-treated group [t(39)=−1.487; p=0.131]. Curiously, the **100 mg/kg NAC-treated group showed a significant total fluid increase** from 25 ml ± 1.23 to 30.78 ± 1.39 ml [t(39)=−3.71; p=0.006] in the post-abstinence period. Finally, as can be seen in Figure 41 B, no significant alteration in the body weight of the animals was detected.

5.1.2.2 Experiment 2.B. Preclinical study in female Wistar rats

The experiment 2.B was performed with 20 female Wistar rats categorised as ADE animals in experiment 1.B. During the fourth deprivation period, these animals were randomly distributed between two experimental groups treated with **vehicle or NAC 60 mg/kg.** Data of two animals had to be discarded because significant leaks were detected in their bottles at the end of the experiment. Consequently, each experimental group had a size of 10 subjects.

Figure 42 shows the daily time course (left panel) and the 4-day average intake of ethanol (right panel) before (basal) and after (post-abstinence) the deprivation period that lasted 21 days.

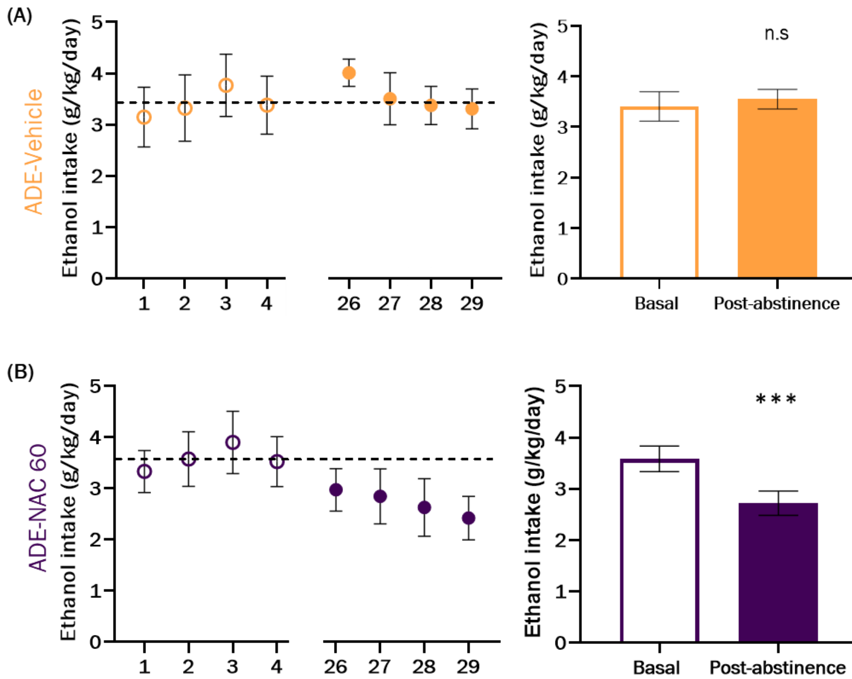


Figure 42. Effect of the subcutaneous injections of NAC on ADE female animals, once a day for 14 days of ***vehicle*** *(A),* ***NAC 60 mg/kg*** *(B) on ethanol intake (n = 10/group). On the left part of A, and B panels, the mean daily ethanol intake determined during the 4 days of baseline and post-abstinence days is shown. Data were analysed by one-way ANOVA for repeated measures. The dashed line represents the mean baseline value. 1, 2, 3, and 4 represent the baseline measurements taken throughout 4 days prior to the abstinence period whereas 26, 27, 28 and 29 represent the post-abstinence measurements. On the right part of panels A, and B, the collapsed values of alcohol intake determined during the 4 days of baseline and the post-abstinence period are represented as bars; the results of the paired Student's t test for the ethanol intake are included. Data are represented as mean ± SEM. [*** p<0.001; n.s. p>0.05]*

As represented in Figure 42 each experimental group displayed a different ethanol relapse-like drinking behaviour:

- After the abstinence period, **vehicle-treated female rats** (Figure 42 A) **did not display a significant change in ethanol intake** in comparison with that observed in the baseline period, as confirmed through two statistical analyses. On one hand, the one-way ANOVA for repeated measures did not find any variation on daily ethanol intake over time [F(7.63)=0.912; p=0.503]. On the other hand, collapsed values of alcohol intake determined during the 4 days of baseline and the post-abstinence period were compared through a paired Student's t-test and no significant differences were detected [t(39)=-0.612; p= 0.542].

- In the case of **female rats treated with NAC 60 mg/kg** (Figure 42 B), **a decrease in ethanol intake after the deprivation period** was observed. Statistical analysis confirmed that the effect on daily ethanol intake over time was statistically significant [F(7,63)=3.680; p=0.002]. Moreover, collapsed values of alcohol intake determined during the 4 days of baseline and the post-abstinence period were compared through a paired Student's t-test and significant differences were found [t(39)=4.101; p<0.001]

In addition, the possible effect of NAC treatment on fluid or food consumption was evaluated. Hence, total fluid intake and animal weight before and after treatment were measured and compared. These data are collected in Figure 43.

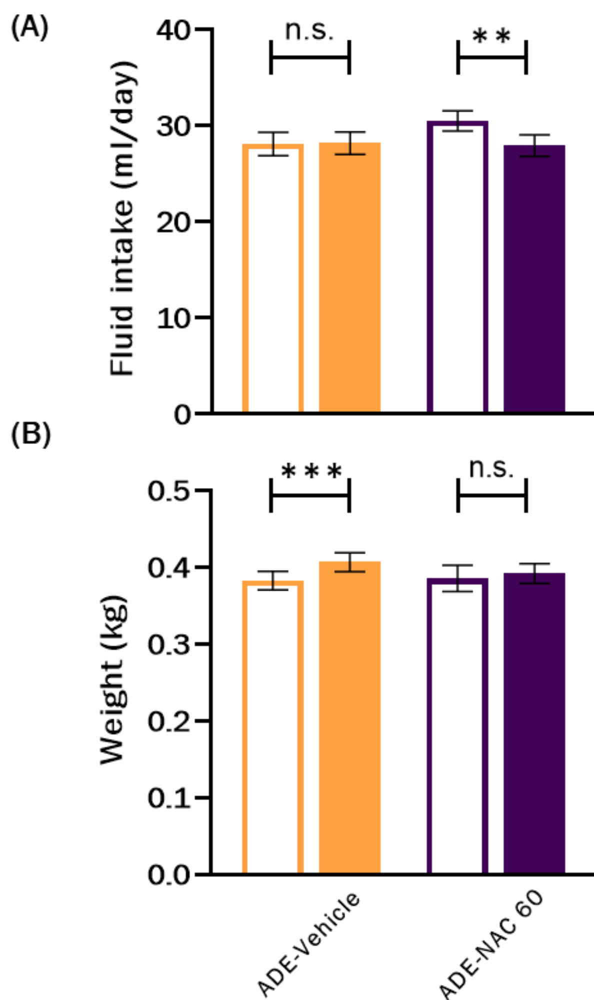


Figure 43. Effect of the treatment on ***fluid intake*** *(A) and* ***body weight*** *(B) of female animals. Data is represented as mean with SEM. Paired Student's t-test was applied [*** p<0.001; n.s. p>0.05]*

The analysis of fluid intake revealed that neither the vehicle [t(39)=0.309; p=0.759] nor the NAC 60 mg/kg treatment

[t(39)=1.870; p=0.069] altered this parameter. Conversely, only animals treated with vehicle had a statistically significant weight increase [t(9)=-6.459; p=0.0001] (Figure 43).

5.1.3 Experiment 3. Brain oxidative and neuroinflammatory status determined in male Wistar rats after 21 days of ethanol abstinence. Effects of alcohol reintroduction

According to the ethanol relapse-like drinking behaviour displayed by our rats in experiment 1, two different subpopulations (ADE and no-ADE) were identified. The aim of experiment 3 was to explore if there are biochemical differences between both subpopulations when animals are experiencing a protracted abstinence. This experiment was designed with the aim of addressing the following questions:

- Does prolonged abstinence trigger brain oxidation or neuroinflammatory imbalances?
- If so, at these biochemical levels, do any differences exist between both subpopulations?
- What is the role of ethanol when it is reintroduced during 24 hours?

In order to answer these questions, two different sub experiments were performed:

Experiment 3.1. Analysis of the oxidative status in HIP and AMG of the male rats.

Experiment 3.2. Determination of several neuroinflammatory mediators in PFC of the male rats.

5.1.3.1 EXPERIMENT 3.1. ANALYSIS OF OXIDATIVE STATUS

In order to analyse brain oxidative status, GSH and GSSG levels were analysed in HIP and AMG by UPLC-MS/MS. Furthermore, GSSG/GSH ratio, which is one of the most common markers of oxidative stress, was also calculated in order to facilitate the interpretation of the results.

Figure 44 includes the levels of GSSG (A), GSH (B) and GSSG/GSH ratio (C) determined in the HIP of our rats under different experimental conditions.

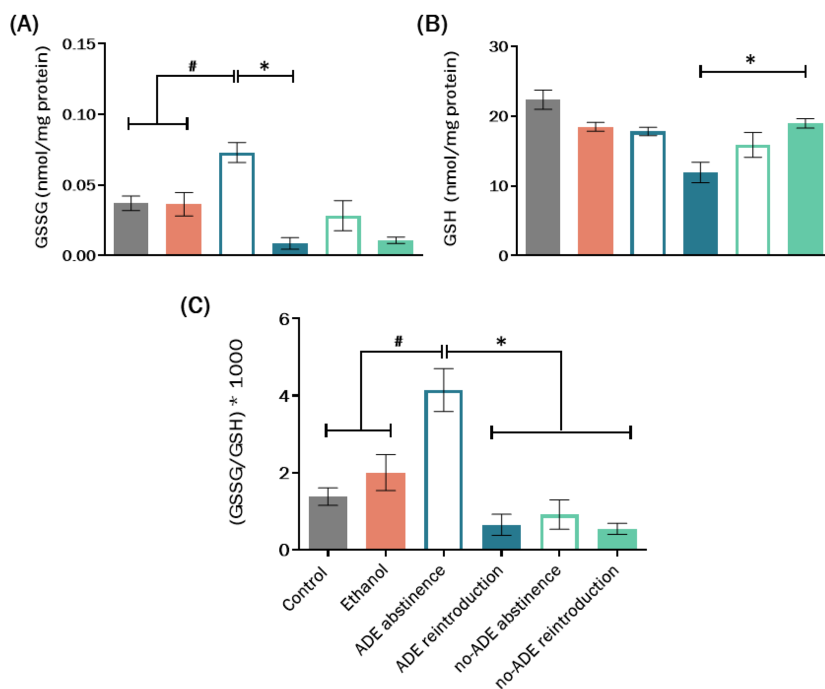


*Figure 44. **GSSG** (A) and **GSH** (B) levels, both expressed as nmol/mg protein, and **GSSG/GSH ratio** (C) determined in the **HIP** of male Wistar rats under different experimental conditions: animals chronically exposed to water consumption (control; solid grey); animals chronically exposed to ethanol*

consumption (ethanol; solid red), ADE animals under abstinence conditions (ADE abstinence; framed blue bar); ADE animals after 24 hours of ethanol reintroduction (ADE reintroduction; solid blue bar); no-ADE animals under abstinence (no-ADE abstinence; framed green bar) and no-ADE animals after 24 hours of ethanol reintroduction (no-ADE reintroduction; solid green bar). Data are represented as mean ± SEM. One-way ANOVA results are represented. Asterisk (*) indicates significant differences among groups, and the hash signal (#) indicates significant differences with respect to control or ethanol groups.

When **hippocampal GSSG** or **GSH** levels were compared among the different experimental conditions (Figure 44 A and B), one-way ANOVA detected statistical differences in both cases. Statistical results were [$F(5,28)=8.523$; $p<0.0001$] and [$F(5,27)=8.320$; $p<0.0001$], respectively. Very interestingly, when **hippocampal GSSG/GSH ratios** were analysed (Figure 44 C), one-way ANOVA also detected statistical differences [$F(5,24)=13.77$; $p<0.0001$]. Among the large amount of obtained data, the most remarkable results were:

- As can be observed in Figure 44 C, under our experimental conditions, animals with long-term exposure to **voluntary ethanol consumption** did not show statistical differences with respect to control animals when the GSSG/GSH was analysed.

- A large difference between the **ADE** and **no-ADE** groups in the GSSG/GSH ratio was detected when animals were subjected to a **21-day deprivation period.** Particularly, as can be observed in Figure 44 C, rats of the ADE group experienced a great increase in oxidative stress levels during abstinence (4.147 ± 0.557) with respect to the control group (1.378 ± 0.227; $p<0.0001$) or ethanol exposed rats (2.005 ± 0.468; $p=0.005$). However, the GSSG/GSH

ratio in no-ADE rats (0.917 ± 0.382) remained invariable with respect to control and ethanol groups during the abstinence period. Additionally, Tukey's post-hoc test also detected statistical differences in this ratio between the ADE and no-ADE abstinence groups ($p<0.0001$).

- After 24 hours of **ethanol reintroduction**, the GSSG/GHH ratio was re-established from 4.147 ± 0.557 to 0.649 ± 0.272 ($p<0.0001$) in the ADE group.

- In general, **GSSG levels** displayed **similar trends** as those observed in the GSSG/GSH ratio. Thus, during abstinence, a marked and significant increase in GSSG is noted only in ADE rats (0.073 ± 0.007 nmol/mg protein), when compared to either the control (0.037 ± 0.005 nmol/mg protein; $p=0.022$) or ethanol groups (0.036 ± 0.008 nmol/mg protein; $p=0.043$) (see Figure 44 A). Curiously, the reintroduction of ethanol in abstinent ADE rats rapidly alleviated this rise and restored GSSG levels (0.008 ± 0.004 nmol/ mg protein; $p<0.0001$)

These statistical results were confirmed through an additional analysis. Specifically, two-way ANOVA revealed:

- A significant *subpopulation* [$F(1,14)=28.730$; $p<0.001$], *ethanol reintroduction* [$F(1,14)=38.670$; $p<0.001$] and *ethanol reintroduction x subpopulation* interaction effect [$F(1, 14)=25.210$; $p<0.001$] on **hippocampal GSSG/GSH ratio**.

- A significant *subpopulation* [F(1,15)=9.821; p=0.007*], ethanol reintroduction* [F(1,15)=36.240; p<0.001] and ethano*l reintroduction* x *subpopulation* interaction [F(1,15)=11.990; p=0.003] effects on **hippocampal GSSG** values.

As previously pointed out, the **oxidative status in AMG** was also analysed. Figure 45 represents levels of GSSG (A), GSH (B) and the GSSG/GSH ratio (C) determined in AMG.

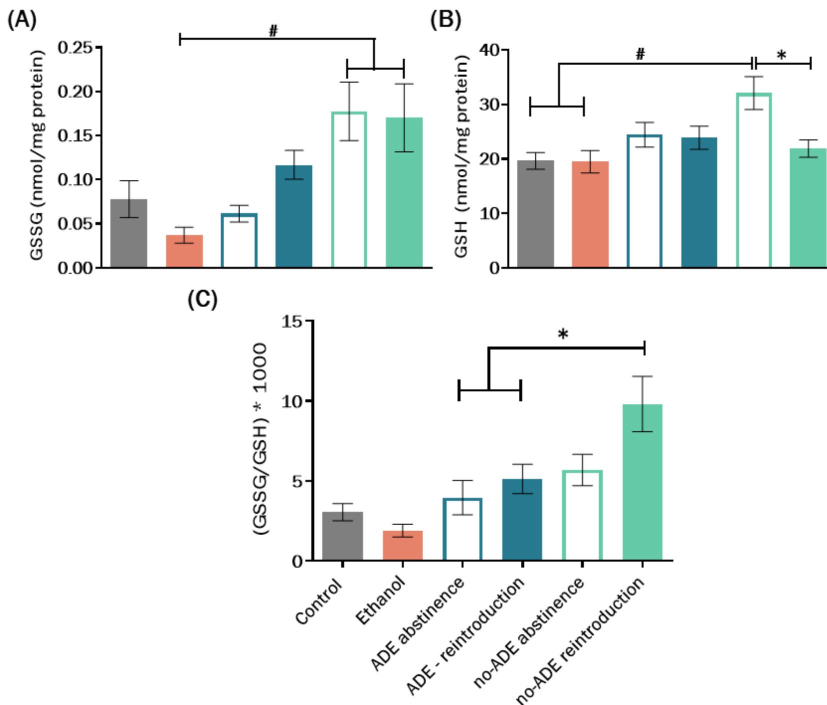


Figure 45. ***GSSG*** *(A) and* ***GSH*** *(B) levels, both expressed as nmol/mg protein, and* ***GSSG/GSH ratio*** *(C) determined in the* ***AMG*** *of male Wistar rats under the experimental conditions previously described in figure 44. Data are represented as mean ± SEM. One-way ANOVA results are represented. Asterisk (*) indicates significant differences among groups, and the hash*

signal (#) indicates significant differences with respect to control or ethanol groups.

When **GSSG** and **GSH** levels were analysed in AMG under the different experimental conditions (Figure 45 A and B) one-way ANOVA found significant differences [$F(5,26)=4.764$; $p=0.003$] and [$F(5,26)=4.997$; $p=0.002$], respectively. Once again, the comparison of **GSSG/GSH ratio** in AMG among groups revealed significant differences [$F(5,22)=8.156$; $p<0.001$]. The most remarkable results were

- As reflected in Figure 45 C, the **GSSG/GSH ratio** did not differ between **ADE** and **no-ADE** groups during **long-term abstinence** in AMG. Besides, **chronic voluntary ethanol intake** did not significantly affect this parameter.

- **GSSG/GSH** values (Figure 45 C) showed that the **no-ADE reintroduction group** had clear differences with respect to the other experimental conditions. Again, the existence of significant differences in GSSG/GSH ratio between the ADE reintroduction (5.138 ± 0.917 nmol/mg) and no-ADE reintroduction (9.821 ± 1.722 nmol/mg protein; $p=0.036$) could be indicative of **differences between the two subpopulations**. The comparison between the no-ADE abstinence group (5.69 ± 0.98 nmol/mg) and no-ADE reintroduction group was almost significant ($p=0.059$), suggesting the potential role of the reintroduction of alcohol in the GSSG/GSH values only in one of the identified subpopulations.

- It is especially remarkable that **during abstinence**, **GSSG levels in no-ADE rats** (0.178 ± 0.033 nmol/mg protein) were significantly higher ($p=0.0136$) when compared with those obtained in ethanol rats (0.037 ± 0.009 nmol/mg protein) (Figure 45 A). Strikingly, these effects were not noticed in ADE rats.

- The increase in GSSG levels detected during **abstinence in no-ADE group** was accompanied by an increase in **GSH levels** (32.07 ± 3.033 nmol/mg protein) when compared with those obtained during the ethanol reintroduction (21.89 ± 1.59 nmol/mg protein; $p=0.018$) (Figure 45 B). However, GSH levels determined during abstinence in AMG did not change in ADE animals.

Moreover, these results were confirmed, at least in part, through a two-way ANOVA analysis, given that:

- A significant effect of *ethanol reintroduction* [$F(1,16)=5.484$; $p=0.033$] was detected on **GSH levels in AMG.**

- A significant *subpopulation* effect [$F(1,15)=7.033$; $p=0.018$] on **GSSG levels was identified in AMG.**

- And a significant *subpopulation* [$F(1,13)=7.117$; $p=0.019$] and *ethanol reintroduction* [$F(1,13)=4.872$; $p=0.045$] effects were revealed through the analysis of the **GSSG/GSH ratio in AMG**.

5.1.3.2 EXPERIMENT 3.2. DETERMINATION OF NEUROINFLAMMATORY MEDIATORS

In order to evaluate neuroinflammatory processes in PFC of ADE and no-ADE rats, the mRNA expression of 7 neuroinflammatory mediators was analysed by RT-qPCR: IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3.

To facilitate data exposure, results obtained in this experiment are presented in 2 different figures. In first place, Figure 46 contains mRNA expression of IL-1β (A), NfκB (B), IL-6 (C) and NLRP3 (D).

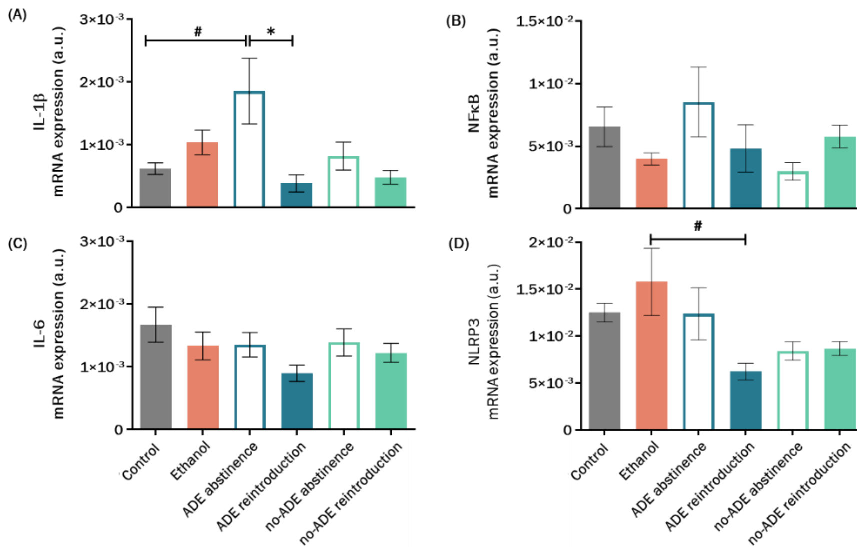


*Figure 46. mRNA expression in **PFC** of **IL-1β**(A), **NfκB** (B), **IL-6** (C) and **NLRP3** (D) of male Wistar rats under the experimental conditions previously described in figure 44. Data are represented as mean ± SEM. One-way ANOVA results are represented. Asterisk (*) indicates significant differences among groups, and the hash signal (#) indicates significant differences with respect to control or ethanol groups.*

The statistical analysis of IL-1β, NfκB, IL-6 and NLRP3 mRNA expression in PFC (Figure 46) only detected significant differences among the compared experimental groups for **IL-1β and NLRP3** expression. One-way ANOVA results were [$F(5,27)=4.696$; $p=0.003$] and [$F(5,25)=3.027$; $p=0.029$], respectively. It is worth highlighting the following results:

- Under our experimental conditions, animals with long-term exposure to **voluntary ethanol consumption** did not show statistical differences with respect to control group in the expression of **IL-1β, NfκB, IL-6** and **NLRP3.**

- As depicted in Figure 46 A, during **abstinence**, the expression of **IL-1β in the ADE** group was increased up to 200% (0.0019 ± 0.0005 a.u.) in comparison with control animals (0.0006 ± 0.00009 a.u.; p=0.014). Moreover, the **reintroduction of alcohol** restored the imbalance (0.0004 ± 0.0001 a.u.; p=0.004). These alterations were detected only in ADE animals, since in no-ADE animals Il-1β levels did not vary during the abstinence period nor the reintroduction of alcohol.

- The **NfκB** mRNA expression showed a similar pattern to the obtained in the analysis of IL-1β. However, no statistical differences were detected (Figure 46 B).

These results were confirmed, at least in part, through a two-way ANOVA analysis:

- A significant *ethanol reintroduction* effect [$F(1,17)=10.090$; $p=0.006$] was found in **IL-1β PFC expression.** Moreover, a nearly significant interaction between *ethanol reintroduction x subpopulation* was detected [$F(1,17)=3.956$; $p=0.063$].

- A significant *ethanol reintroduction* [$F(1,17)=4.864$; $p=0.042$] and *ethanol reintroduction x subpopulation interaction* [$F(1,17)=5.792$; $p=0.028$] effects were found in the analysis of **NLRP3 expression in PFC.**

To continue with the analysis of neuroinflammatory markers in PFC, Figure 47 presents mRNA expression of TNF-α, HMGB1 and iNOS under the same experimental conditions.

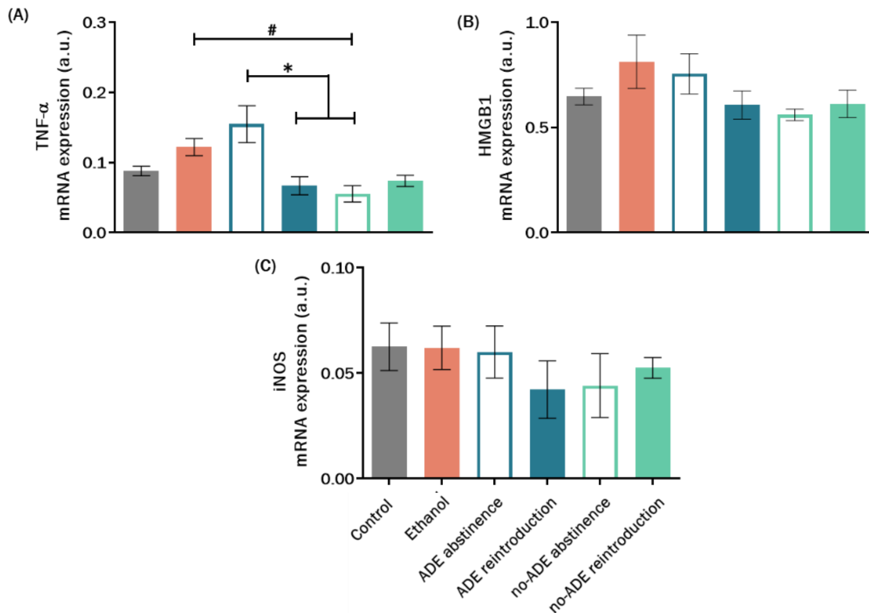


*Figure 47. mRNA expression in **PFC** of **TNF-α** (A), **HMGB1** (B) and **iNOS** (C) of male Wistar rats under the experimental conditions previously described in figure 44. Data are represented as mean ± SEM. One-way ANOVA results are represented. Asterisk (*) indicates significant differences among groups, and the hash signal (#) indicates significant differences with respect to control or ethanol groups.*

We can appreciate in Figure 47 that only **TNF-α** mRNA expression was altered under our experimental conditions in PFC (Figure 47 A). One-way ANOVA result confirmed this observation [$F(5,27)=7.176$; $p<0.001$]. Neither HMGB1 (Figure 47 B) nor iNOS (Figure 47 C) expression was significantly altered under any experimental condition. The most important evidence derived is:

- Under our experimental conditions, animals that were exposed to long-term **voluntary ethanol consumption** did not show statistical

differences with respect to control animals when the **TNF-α, HMGB1** and **iNOS** expression was analysed.

- As depicted in Figure 47 A, **abstinence** differently affected the expression of **TNF-α** in ADE and no-ADE animals. TNF-α mRNA levels in PFC of ADE animals during 21 days of deprivation were significantly higher (0.16 ± 0.026 a.u.) than those observed in no-ADE animals (0.06 ± 0.012 a.u.; $p<0.001$).

- The presence of ethanol after the deprivation period rapidly restored **TNF-α** levels for ADE animals (0.067 ± 0.013 a.u.; $p=0.002$).

The results regarding **TNF-α expression in PFC** were corroborated through an additional two-way ANOVA that revealed:

- A significant *subpopulation* [$F(1,18)=9.118$; $p=0.007$], *ethanol reintroduction* [$F(1,18)=5.147$; $p=0.036$] and *ethanol reintroduction x subpopulation interaction* [$F(1,18)=12.080$; $p=0.003$] effects.

5.1.4 Experiment 4. Unravelling the mechanism of action underlying NAC anti-relapse effect.

The anti-relapse effect of NAC 60 mg/kg and NAC 100 mg/kg in male Wistar rats has been previously demonstrated in experiment 2 of the present thesis. Even so, the underlying mechanism of action has not been explored yet in our experimental conditions. Hence, the purpose of experiment 4 was to explore three potential mechanisms of action by which NAC could exert its ethanol anti-relapse effect. To do so, NAC effects were evaluated always after a protracted ethanol abstinence period (21 days). Experiment 4 is divided into 3 different experiments.

Experiment 4.1. NAC effects on the brain of oxidative status during abstinence.

Experiment 4.2. NAC effects on the expression of several neuroinflammatory mediators during abstinence.

Experiment 4.3. NAC effects on the expression of three glutamatergic proteins (GLT1. GLAST and xCT) during abstinence.

In order to facilitate the interpretation of the results, we must remember that all data in experiment 4 were analysed using two different analyses of variance (one-way ANOVA):

- The influence of ethanol consumption or ethanol abstinence were studied through the comparison among data derived from Control, Ethanol, and ADE Abs-Vehicle group.

- The comparison among data from ADE Abs-Vehicle, ADE Abs-NAC 60 mg/kg and ADE Abs-NAC 100 mg/kg allowed us to explore the effect of NAC under abstinence conditions.

Although data from Control, Ethanol and ADE Abs-Vehicle groups had previously been reported in experiment 3, they have been plotted together with the present results as well as included in the statistical analysis for comparative purposes.

5.1.4.1 Experiment 4.1. NAC effects on the brain of oxidative status during abstinence.

As previously performed in experiment 3, GSH and GSSG levels in HIP and AMG were analysed by UPLC-MS/MS. After that, GSSG/GSH ratio was calculated to facilitate data analysis.

Firstly, Figure 48 depicts the obtained data of GSSG (A), GSH (B) and GSSG/GSH ratio (C) in the HIP of our rats under different experimental conditions.

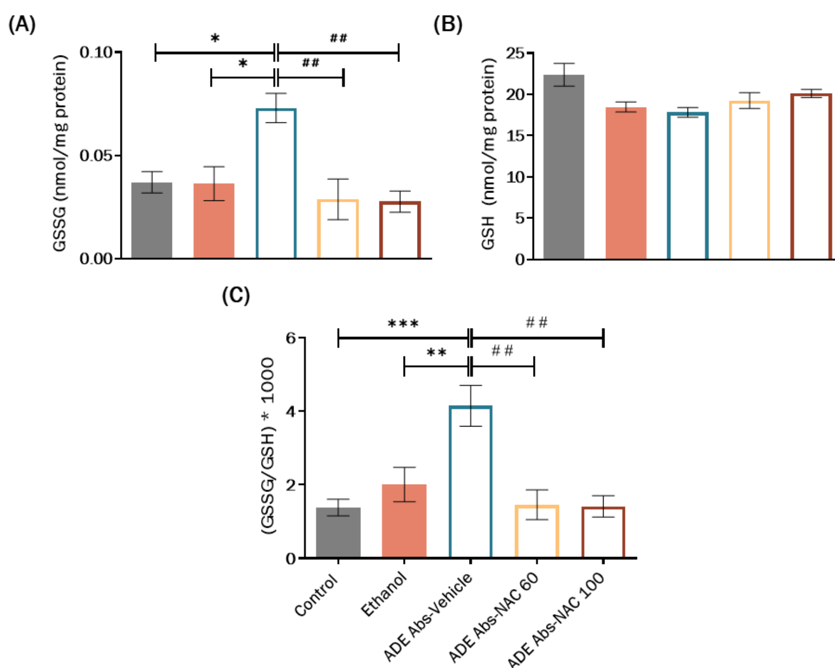


*Figure 48. **GSSG** (A) and **GSH** (B) levels, both expressed as nmol/mg protein, and **GSSG/GSH ratio** (C) determined in the **HIP** of male Wistar rats under different experimental conditions: animals exposed to water consumption (control; solid grey), animals chronically exposed to ethanol consumption (ethanol; solid red), ADE animals under abstinence conditions and treated with vehicle (ADE Abs-vehicle; framed blue bar), ADE animals in abstinence treated with NAC 60 mg/kg (ADE Abs-NAC 60; framed orange bar) and ADE animals in abstinence treated with NAC 100 mg/kg (ADE Abs-NAC 100; framed burgundy bar). Data are represented as mean ± SEM. The results of two one-way ANOVA analysis are represented. Asterisk (*) indicate the significant differences among control, ethanol and ADE Abs-Vehicle group whereas the hash signal (#) indicates significant differences among ADE Abs-treated groups. [## p<0.01; * p< 0.05; ** p<0.01; *** p<0.001].*

The analysis of **GSSG and GSH levels in HIP** revealed that GSH levels were not modified under any of the analysed experimental conditions. Nonetheless, interesting results were found when **GSSG and the**

GSSG/GSH ratio were analysed. The most relevant results among the obtained data were:

- As previously found in experiment 3.1, the analysis of hippocampal GSH levels revealed that neither chronic ethanol consumption nor abstinence modified GSH levels [$F(2,15)=3.823$; $p=0.083$]. Curiously, despite the administration of NAC, a theoretical substrate for **GSH** synthesis, **neither NAC 60 mg/kg nor NAC 100 mg/kg caused a significant increase in GSH levels in the HIP** [$F(2,9)=2.495$; $P=0.137$] (Figure 48 B)

- Similar to experiment 3, after 21 days of ethanol deprivation, **GSSG** levels were significantly increased in the HIP [$F(2,15)=5.768$; $p=0.014$] when compared with the control ($p=0.016$) or ethanol ($p=0.02$) group (Figure 48 A). Interestingly, **when animals were treated with NAC during abstinence, GSSG values were restored** [$F(2,9)=10.27$; $p=0.005$]. In particular, animals treated with NAC 60 mg/kg and 100 mg/kg doses showed significant differences with respect to the vehicle-treated group (0.029 ± 0.01 nmol/ mg protein; $p=0.009$) and (0.028 ± 0.005 nmol/ mg protein; $p=0.006$), respectively.

- The analysis of **GSSG/GSH ratio** confirmed again that NAC was able to restore oxidative stress levels during abstinence (Figure 48 C). During this stage the GSSG/GSH ratio was significantly increased [$F(2,12)=13.62$; $p<0.001$] and rose to 300% ($p<0.001$) and 207% ($p<0.01$) in comparison with control and ethanol groups, respectively. Exactly, the GSSG/GSH ratio reached a value of 4.147

± 0.557 nmol/ mg protein, but the treatment with NAC was able to restore it [F(2,9)=13.50; p=0.002] after the administration of both NAC doses: 60 mg/kg (1.453 ± 0.406; p=0.004) and 100 mg/kg (1.409 ± 0.292; p=0.003).

As previously commented, **oxidative status in the AMG** was also analysed. Figure 49 summarises levels of GSSG (A), GSH (B) and the GSSG/GSH ratio (C) determined in the AMG under the different experimental conditions assayed.

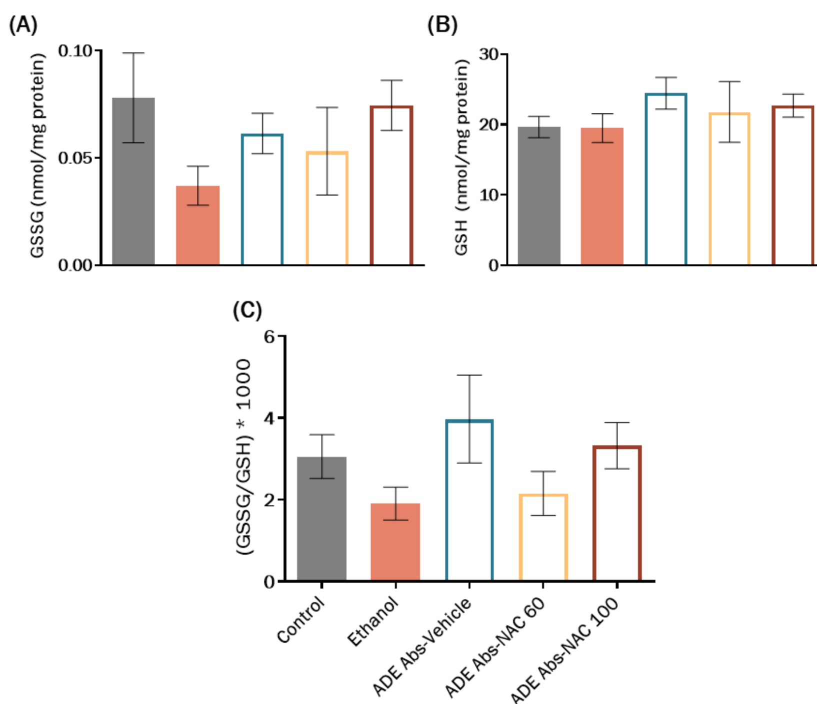


*Figure 49. **GSSG** (A) and **GSH** (B) levels, both expressed as nmol/mg protein, and **GSSG/GSH ratio** (C) determined in the **AMG** of male rats under the experimental conditions previously described in figure 48. Data are represented as mean ± SEM.*

Concerning data obtained in the AMG, **one-way ANOVA did not detect statistical differences** when GSSG [F(2,14)=1.325, p=0.297], GSH [F(2,13)=1.900, p=0.189] or GSSG/GSH ratio [F(2,12)=2.254, p=0.148] were analysed after chronic alcohol exposition or ethanol abstinence. Moreover, NAC administration did not modify neither GSSG [F(2,10)=0.600, p=0.568] nor GSH [F(2,10)=0.215, p=0.810] nor GSSG/GSH ratio [F(2,10)=2.254, p=0.148].

Overall, these findings suggest that under our experimental conditions, oxidative status is only altered in HIP during abstinence and both NAC doses, chronically administered during this period, are able to alleviate this imbalance, leading it to basal conditions.

5.1.4.2. Experiment 4.2. NAC effects on the expression of several neuroinflammatory mediators during abstinence.

The measurement of mRNA expression of inflammatory mediators such as IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3 by RT-PCR was used to evaluate the neuroinflammatory processes in PFC during abstinence and the potential effects of NAC on them.

Obtained results are summarised in Figure 50 and Figure 51. Firstly, Figure 50 shows the results of mRNA expression of IL-1β (A), NfκB (B), IL-6 (C) and NLRP3 (D) under the different experimental conditions.

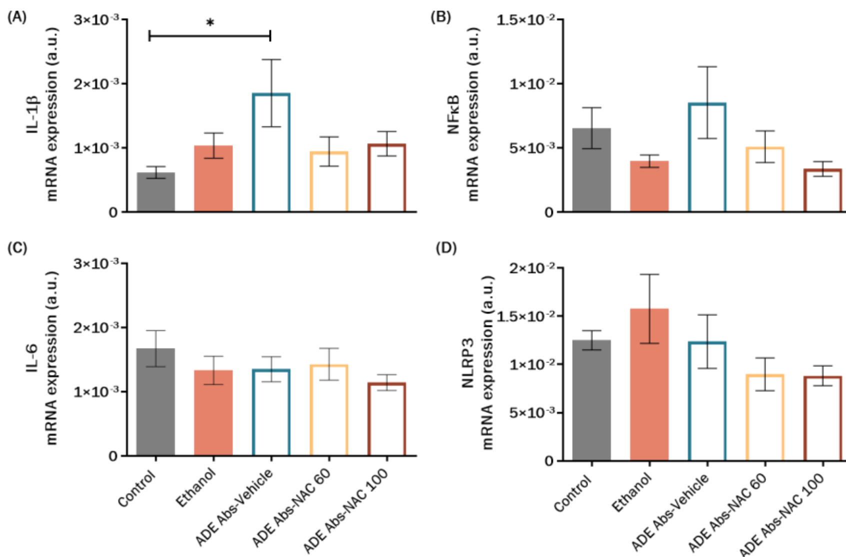


*Figure 50. mRNA expression of **IL-1β** (A), **NfκB** (B), **IL-6** (C) and **NLRP3** (D) measured in the **PFC** of male Wistar rats under the experimental conditions previously described in figure 48. Data are represented as mean ± SEM. The results of one-way ANOVA analysis are represented. Asterisk (*) indicate the significant differences among control, ethanol and ADE Abs-Vehicle group [* $p < 0.05$].*

As can be appreciated in Figure 50, the one-way ANOVA test only detected statistical differences in **the expression of IL-1β** when control, ethanol and abstinence conditions were compared [$F(2,14)=4.322$; $p=0.035$]. The mRNA levels of NfκB, IL-6 and NLRP3 did not significantly change among the different experimental conditions assayed (Figure 50 B, C and D). The most important findings are summarised below:

- Similar to experiment 3, during **abstinence**, the expression of **IL-1β** was increased up to 200% (0.0019 ± 0.0005 a.u.; $p=0.029$) in comparison with control animals (0.0006 ± 0.00009 a.u.).

- The data plotted in Figure 50 A demonstrates that ADE animals under abstinence conditions that were treated with NAC 60 mg/kg or NAC 100 mg/kg (0.0009 ± 0.0002 a.u.) (0.0011 ± 0.0002 a.u.) did not suffer an increase of this neuroinflammatory modulator during abstinence. This observation suggests that **NAC treatment seems to prevent the up-regulation of IL-1β expression detected in the ADE Abs-vehicle group.** Statistical analysis was not able to detect significant differences when vehicle, NAC 60 and NAC 100 treated groups were compared [$F(2,12)=2.025$; $p=0.175$].

- Even though the profile of **NfκB** results is quite similar to the obtained with IL-1β (Figure 50 B), no statistical differences were found.

Figure 51 presents mRNA expression of TNF-α (A), HMGB1 (B) and iNOS (C).

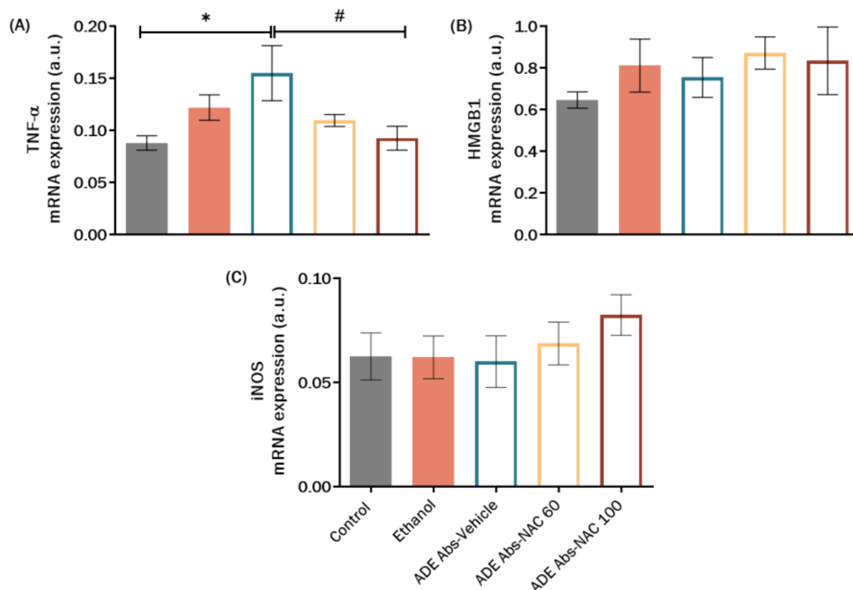


*Figure 51. mRNA expression in **PFC** of **TNF-α** (A), **HMGB1** (B) and **iNOS** (C) measured in the **PFC** of male Wistar rats under the experimental conditions previously described in figure 49. Data are represented as mean ± SEM. The results of two one-way ANOVA analysis are represented. Asterisk (*) indicate the significant differences among control, ethanol and ADE Abs-Vehicle group whereas the hash signal (#) indicates significant differences among ADE Abs-treated groups [* p< 0.05; # p< 0.05].*

As depicted in Figure 51, only TNF-α mRNA levels were significantly altered by the experimental conditions assayed. Concerning HMGB1 (Figure 51 B) and iNOS (Figure 51 C), their expression was not significantly modified under any experimental condition. The most remarkable results were:

- The one-way ANOVA analysis [$F(2,13)=4.517$; $p=0.032$] revealed that ADE animals under abstinence conditions had increased levels of TNF-α mRNA levels (0.155 ± 0.026 a.u.) in comparison with control group ($p=0.026$). Nonetheless, NAC treatment was able to prevent this increase [$F(2,12)=4.830$; $p=0.029$]. Concretely, the *post hoc* analysis revealed significant differences between vehicle and **NAC 100 mg/kg treatment** group (0.093 ± 0.120 a.u; $p=0.027$).

5.1.4.3 NAC EFFECTS ON THE EXPRESSION OF THREE GLUTAMATERGIC PROTEINS (GLT1, GLAST AND XCT) DURING ABSTINENCE.

The evaluation of the expression of several proteins involved in the regulation of glutamate levels was the selected approximation to elucidate the potential effect of NAC treatment on glutamate homeostasis in striatum during long-term abstinence. The proteins studied were GLT1, GLAST and xCT (the catalytic subunit of system xC). Thus, the expression of these proteins was analysed in two striatal regions: the DS and the nucleus accumbens through Western Blot technique.

The total protein expression of GLT1 (A) GLAST (B) and xCT (C) in nucleus accumbens is depicted in Figure 52.

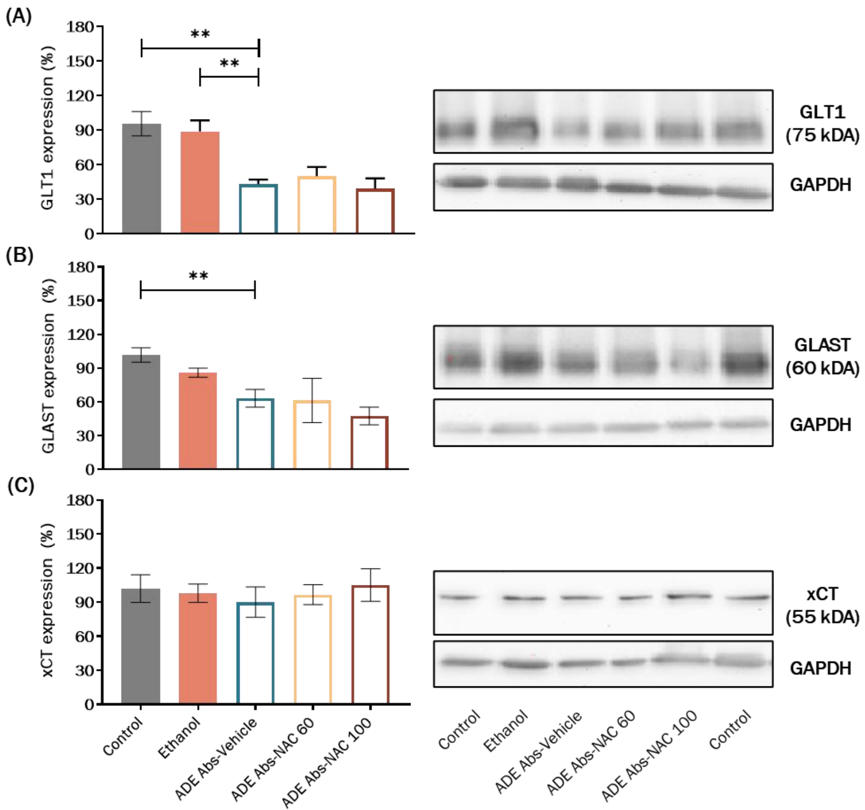


*Figure 52. Total protein expression of **GLT1** (A), **GLAST** (B) and **xCT** (C) (expressed in (%) of control group) measured in the **nucleus accumbens** of male Wistar rats under the experimental conditions previously described in figure 48. The right panel shows representative images obtained with each primary antibody. Data are represented as mean ± SEM. The results of two one-way ANOVA analysis are represented. Asterisk (*) indicate the significant differences among control, ethanol and ADE Abs-Vehicle group whereas the hash signal (#) indicates significant differences among ADE Abs-treated groups [** p< 0.01].*

As can be observed in Figure 52, when comparing the protein expression of GLT1, GLAST and xCT in nucleus accumbens, one-way ANOVA revealed significant differences among the different

experimental groups for GLT1 and GLAST but not for xCT. The most important results were:

- One-way ANOVA revealed significant differences in GLT1 when its expression in control, ethanol and ADE Abs-Vehicle groups was compared [F(2,14)=9.433; p=0.003] (Figure 52 A). **The protein expression of GLT1 is diminished during abstinence** in nucleus accumbens (0.43 ± 0.04 %) in comparison to control (0.96 ± 0.11 %.; p=0.003) and ethanol group (0.89 ± 0.10 %; p=0.009). Conversely, chronic exposure to ethanol did not affect the expression of GLT1 when compared with respect to the control group (p=0.854). Neither the treatment with **NAC 60 mg/kg** nor **NAC 100 mg/kg** were able to prevent the diminution of GLT1 levels observed during abstinence in nucleus accumbens [F(2,11)=0.608; p=0.562].

- Similarly, **GLAST** expression varied when it was compared between control, ethanol and ADE Abs-Vehicle groups in nucleus accumbens [F(2,14)=9.434; p=0.003] (Figure 52 B). A significant **reduction of around 37% was detected under abstinence conditions** (0.63 ± 0.01 %) in comparison to control group (1.01 ± 0.07 %; p=0.002). Nonetheless, after **chronic ethanol consumption** the protein expression of GLAST remained invariable (p=0.066). As in the case of GLT1, neither the treatment with **NAC 60 mg/kg** nor **NAC 100 mg/kg** was able to prevent the downregulation of GLAST expression in nucleus accumbens during abstinence [F(2,11)=1.848: p=0.203].

- Concerning **xCT** expression in nucleus accumbens (Figure 52 C), no statistical differences were detected after chronic ethanol intake or alcohol abstinence [F(2,13;=0.280; p=0.760]. Similarly, NAC treatment did not alter the expression of xCT [F(2,11)=0.368; p=0.701].

The protein expression results of GLT1 (A), GLAST (B) and xCT (C) determined in DS are represented in Figure 53.

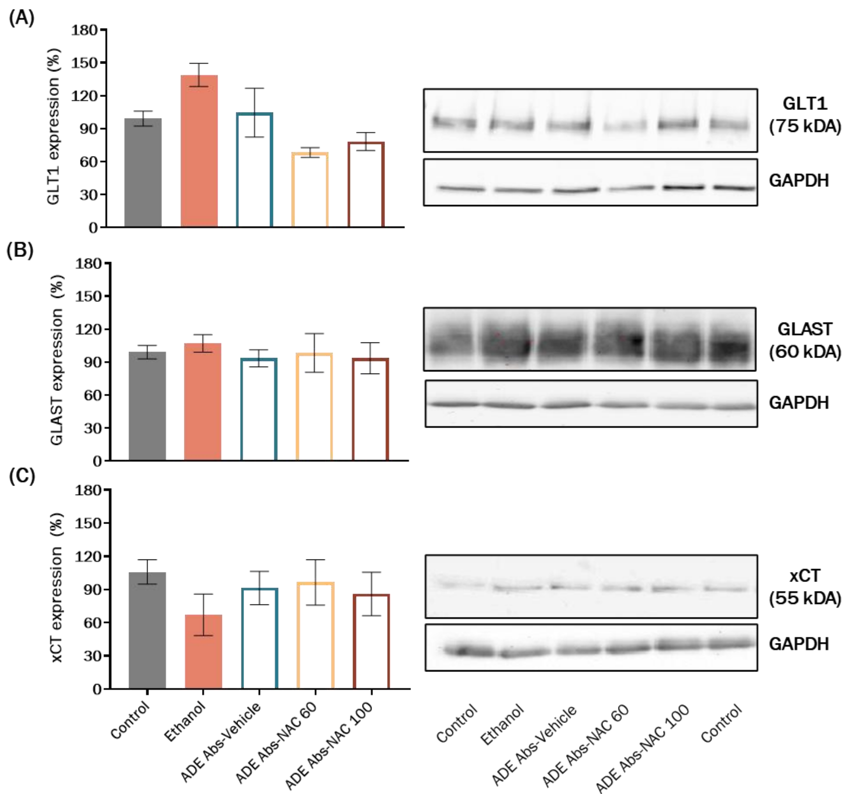


*Figure 53. Total protein expression of **GLT1** (A), **GLAST** (B) and **xCT** (C) (expressed in (%) of control group) measured in the **DS** of male Wistar rats under the experimental conditions previously described in figure 48. The right*

panel shows representative images obtained with each primary antibody. Data are represented as mean ± SEM.

As can be observed in Figure 53, the comparison of GLT1 [$F(2,14)=2.633$; $p=0.107$], GLAST [$F(2,14)=0.851$; $p=0.448$] and xCT [$F(2,14)=1.717$; $p=0.215$] levels in DS through one-way ANOVA analysis did not find any significant differences. Similarly, NAC treatment neither changes the expression of GLT1 [$F(2,11)=1.569$; $p=0.252$], nor GLAST [$F(2,11)=0.042$; $p=0.959$], nor xCT [$F(2,11)=0.077$; $p=0.926$]

5.2. Acute effects of NAC in the MCLS

5.2.1 Experiment 5. Acute effect of NAC on mesocorticolimbic system activation

The aim of experiment 5 was to explore the acute effects of NAC within the rat MCLS. Specifically, we explored the effect of a low and a high dose of NAC (30 and 100 mg/kg) in the MCLS activation (specifically in nucleus accumbens) and the role that mGluR5 could play in this activation. Additionally, NAC's effects on the ethanol-induced activation of the MCLS were tested. To address these issues two experiments were designed.

Experiment 5.1. The efficacy of MTEP (a selective negative allosteric modulator of mGluR5) to suppress the activation of the MCLS induced by NAC.

Experiment 5.2. The effect of 120 mg/kg of NAC on the ethanol-induced activation of the MCLS.

In both cases, cFOS-IR cells counting in nucleus accumbens was used as a direct measure of MCLS activation. Furthermore, experiment 5.2 included a locomotor activity test as an indirect measure of the MCLS activation.

5.2.1.1 Experiment 5.1. The efficacy of MTEP to suppress the activation of mesocorticolimbic system induced by NAC.

The number of cFOS-IR cells in nucleus accumbens was analysed in order to evaluate the effect of low (30 mg/kg) and high (120 mg/kg) doses of NAC on the activation of MCLS. Furthermore, MTEP (0.1

mg/kg) was used to investigate the role of mGluR5 receptors in this activation process.

Representative images of the coronal sections after cFOS staining in the nucleus accumbens, under different experimental conditions, are shown in Figure 54.

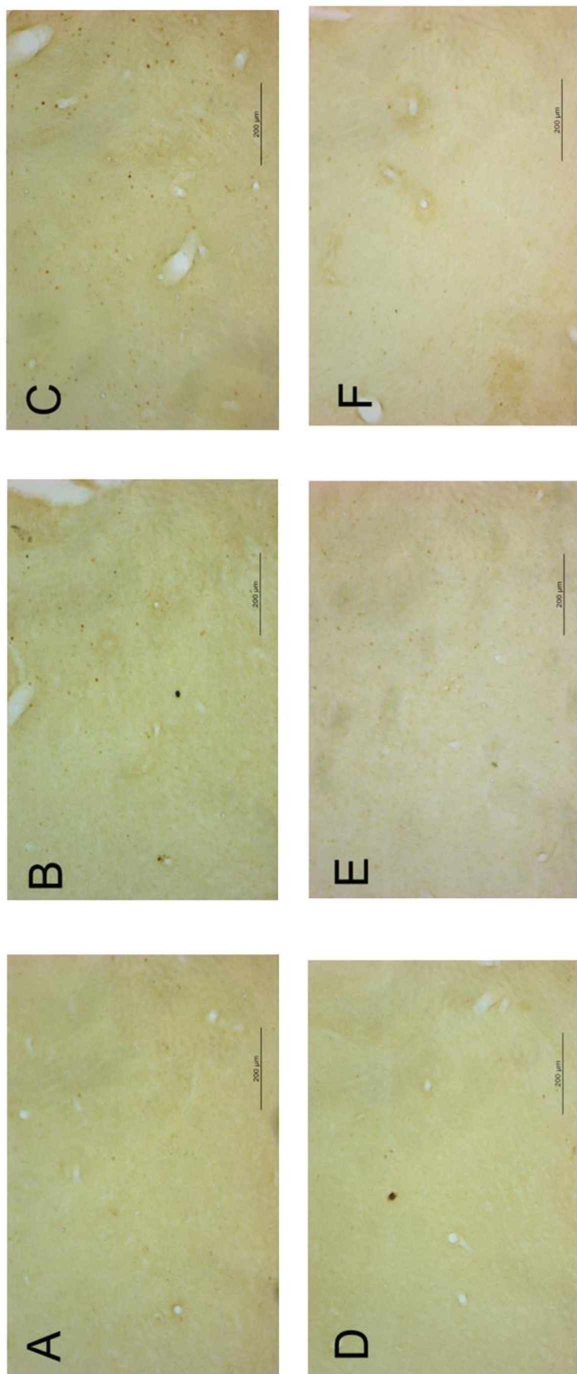


Figure 54. Representative images of coronal sections of ***nucleus accumbens*** *after* ***cFOS staining*** *in experiment 5.1. (A)* ***Veh/Sal*** *group; (B)* ***NAC 30/Sal*** *group; (C)* ***NAC 120/Sal*** *group; (D)* ***Veh/MTEP*** *group; (E) group* ***NAC 30/MTEP*** *group and (F)* ***NAC 120/MTEP*** *group.*

The number of cFOS-IR cells quantified at each experimental group are shown in Figure 55.

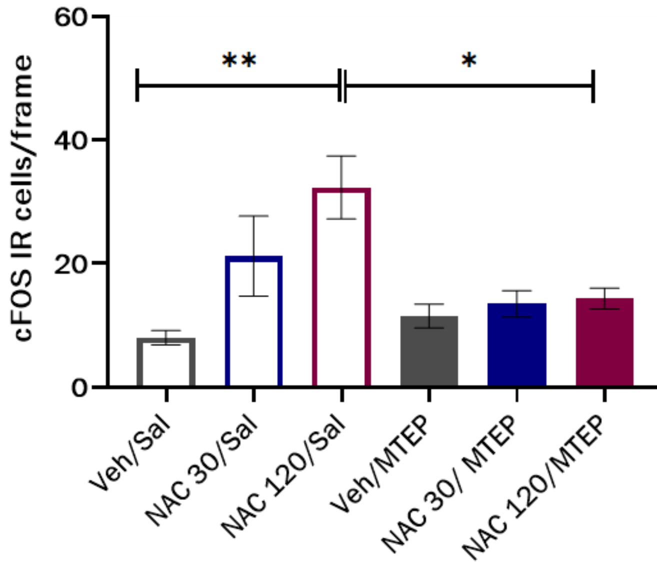


*Figure 55. Effect of **MTEP administration** on cFOS-IR cell expression in nucleus accumbens, induced by low (30 mg/kg) and high doses (120 mg/kg) of NAC. The Y axis represents the number of cells per frame. Grey bars represent vehicle treated groups, blue bars represent NAC 30 mg/kg treated groups and purple bars represent NAC 120 mg/kg groups. Framed bars represent the absence of MTEP whereas solid bars represent the presence of MTEP. Data are represented as mean ± SEM. One-way ANOVA test was performed [* $p < 0.05$; ** $p < 0.01$].*

Figure 55 reflects differences in cFOS-IR cells in nucleus accumbens under our experimental conditions. The result of one-Way ANOVA for this comparison was [$F(5,27)=5.807$; $p<0.001$]. The most remarkable results were:

- The i.p. injection of 30 mg/kg of NAC increased the expression of cFOS-IR cells in nucleus accumbens (21.2 ± 6.5) compared to the vehicle-treated group (8 ± 1.2). However, no statistical differences between groups were detected ($p=0.227$). On the contrary, the administration of a higher dose of **NAC (120 mg/kg) increased their expression** in comparison to the Veh/Sal group (32.33 ± 5.09; $p=0.002$).

- Interestingly, the presence of **MTEP selectively abolished** this increment caused by NAC 120 mg/kg ($p=0.013$), i.e., it restored the number of cFOS-IR cells. These data indicate the efficacy of MTEP in blocking the expression of cFOS-IR cells triggered by the high dose of NAC assayed in our experimental conditions.

5.2.1.2 Experiment 5.2. The Effect of 120 Mg/kg of NAC on the Ethanol-Induced Activation of the Mesocorticolimbic System

In experiment 5.2 we explored the effects of a high NAC dose (i.p., 120 mg/kg) on the activation of the MCLS induced via the intra-VTA administration of 150 nmol of ethanol. The effects were measured through behavioural (studied by the locomotor activity displayed by rats) and neurochemical (determined by counting cFOS-IR cells) assays.

The cannula placements after the histological evaluation of the rat brains used in this experiment are shown in Figure 56. All animals included in experiment 5.2 showed a correct position of the cannula tip in the posterior VTA (between -6.72 mm and -5.64 mm from bregma).

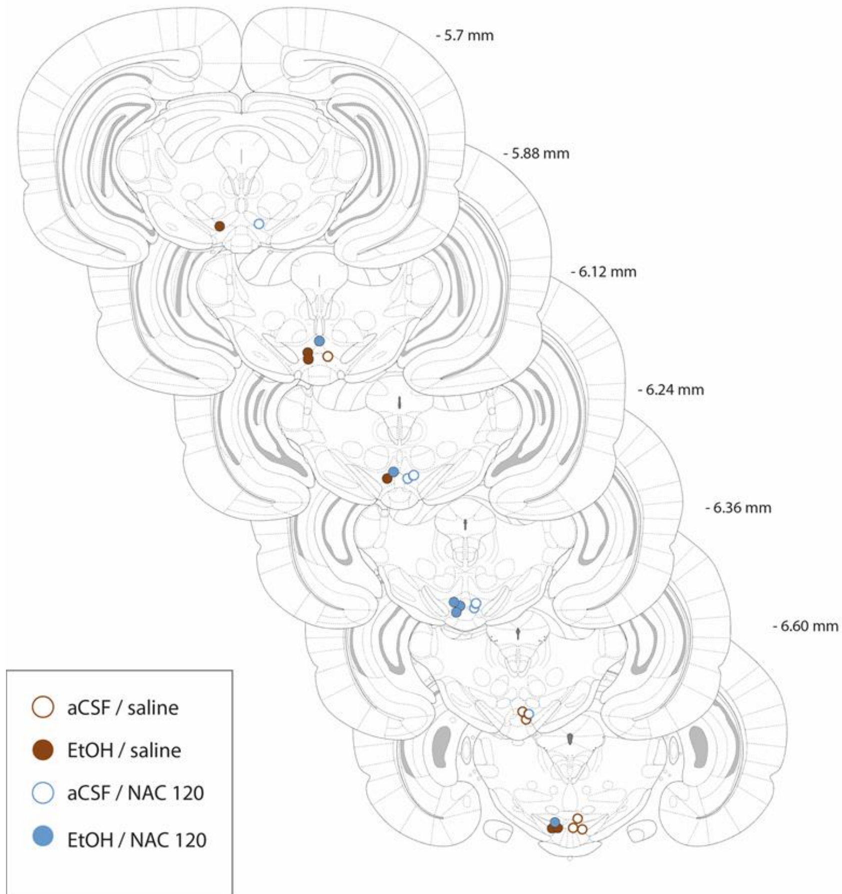


Figure 56. Diagram of coronal sections from the brains of rats used in experiment 5.2, indicating the ***placement of the tip of the injection cannula*** *in the posterior VTA. Numbers indicate the distance from the anterior coordinate to the bregma. Adapted from Paxinos and Watson, 2007.*

Firstly, the activation of MCLS was indirectly measured by a locomotor activity test. The mean values of the distance travelled by each experimental group are depicted in Figure 57.

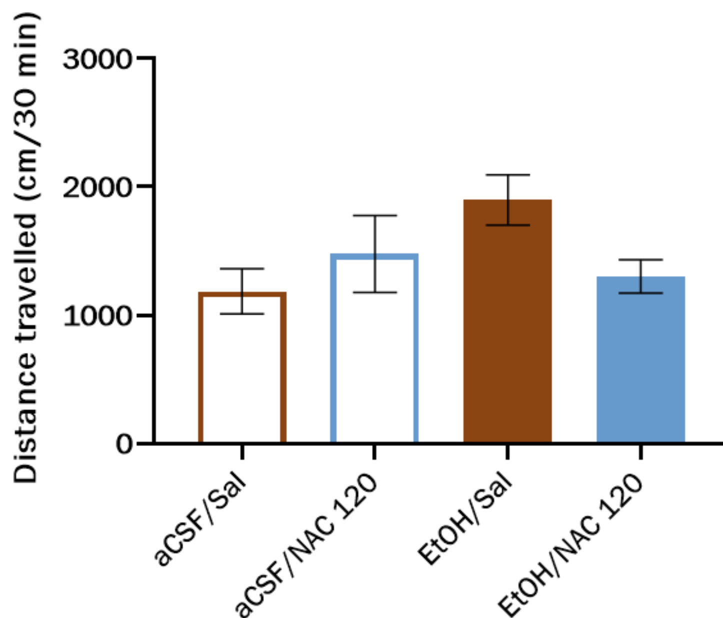


*Figure 57. The effects of **NAC i.p. administration** (120 mg/kg), 30 min prior to the intra-VTA administration of **ethanol** (150 nmol), on the **distance travelled by rats** (cm/30 min). Brown bars represent groups treated with saline while blue bars represent groups treated with NAC 120 mg/kg. Solid bars represent the groups that received ethanol intra-VTA. Data are represented as mean ± SEM. Kruskal-Wallis test did not find any difference between groups.*

As can be observed in Figure 57, the intra-VTA infusion of 150 nmol of ethanol clearly tends toward the increase in the locomotor activity of animals, with respect to the aCSF/Sal group (records increased from 1183 ± 175 up to 1897 ± 196 cm). In addition, i.p. pre-treatment with 120 mg/kg of NAC also showed a trend to attenuate the above-mentioned increase in the locomotor activity induced by ethanol. However, the non-parametric Kruskal–Wallis test did not detect statistically significant differences (p=0.112).

Representative images of coronal sections after cFOS staining in the nucleus accumbens are shown in Figure 58.

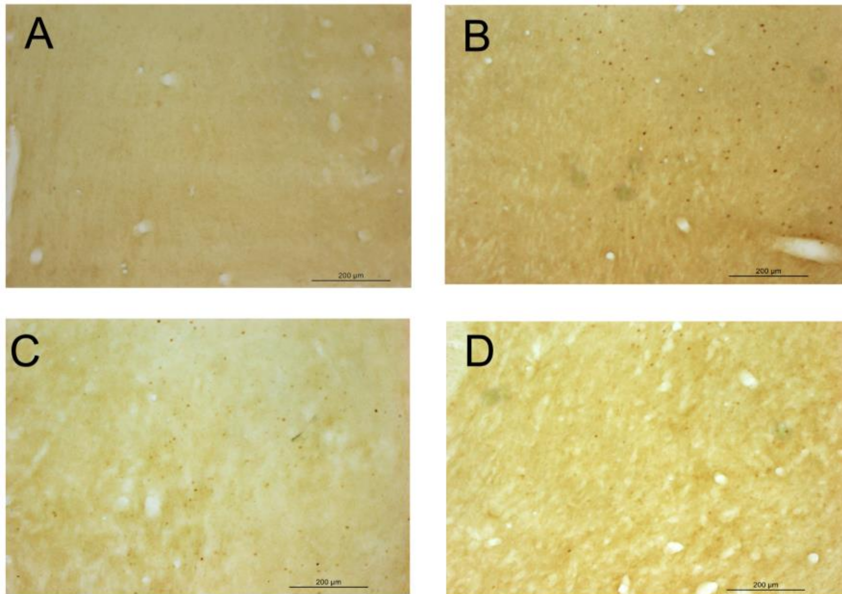


Figure 58. Representative images of coronal sections of ***nucleus accumbens*** *after* ***cFOS staining*** *in experiment 5.2. (A)* ***aCSF/Sal*** *group; (B)* ***aCSF/NAC 120*** *group; (C)* ***EtOH/Sal*** *group and (D)* ***EtOH/NAC 120*** *group.*

Additionally, the activation of the MCLS was directly analysed by quantifying the number of cFOS-IR cells in nucleus accumbens. The experimental results are summarised in Figure 59.

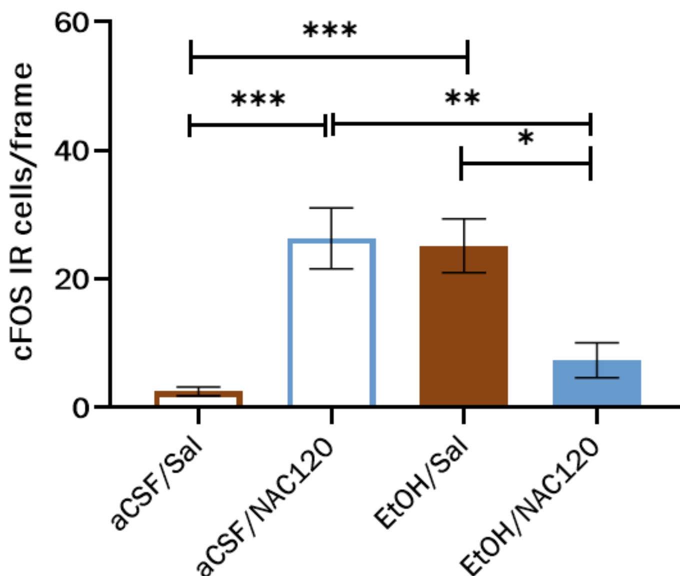


*Figure 59. Effect of **NAC administration** (120 Mg/kg) on **cFOS-IR cell** expression in the nucleus accumbens, 30 minutes prior to the intra-VTA administration of **ethanol** (150 nmol). Brown bars represent groups treated with saline while blue bars represent groups treated with NAC 120 mg/kg. Solid bars represent the groups that received ethanol in VTA. The Y axis represents the number of cells per frame. Data is represented as mean ± SEM. One-Way ANOVA test was performed. [* p < 0.05, ** p < 0.01, *** p < 0.001]*

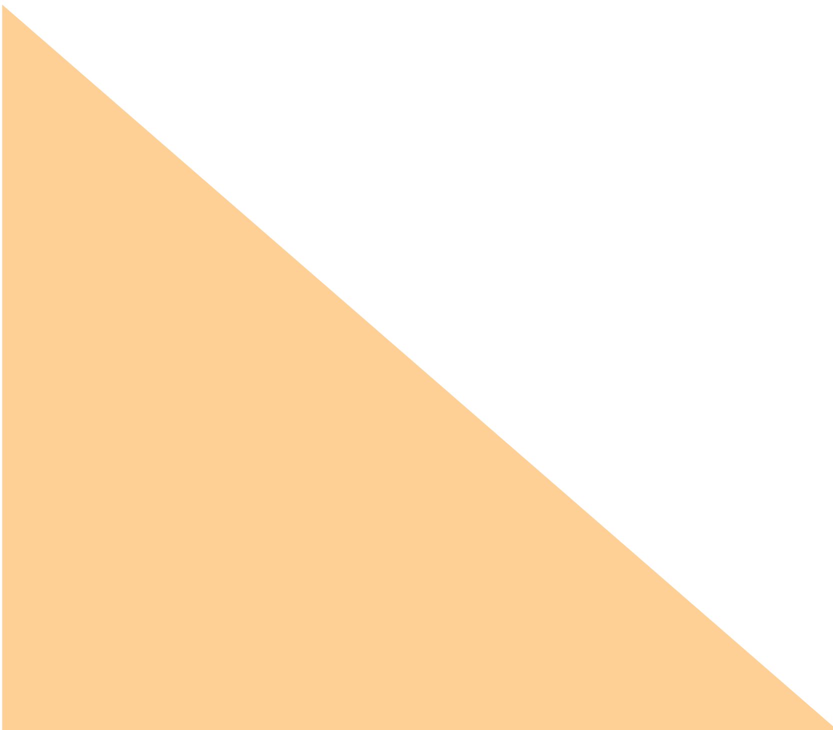
As shown in Figure 59, differences in the number of cFOS-IR cells were found. In particular, one-way ANOVA revealed statistically significant differences among the experimental groups [$F(3,20)=12.36$, $p<0.0001$]. The most remarkable results were:

- On the one hand, the intra-VTA **microinjection of 150 nmol of ethanol caused an augmentation of cFOS-IR cells in nucleus accumbens** with respect to aCSF/Sal group (25.17 ± 4.21;

p<0.001). On the other hand, the administration of **NAC 120 mg/kg triggered a significant increase in the number of cFOS-IR cells in nucleus accumbens** (26.33 ± 4.75) compared to aCSF/Sal (2.5 ± 0.7; p<0.001), similar to experiment 5.1. Hence, these data suggests that NAC 120 mg/kg and 150 nmol of ethanol had the ability to activate the MCLS.

- Very curiously, the **co-administration** of 150 nmol of ethanol (intra-VTA injected) and NAC 120 mg/kg (i.p. injected) totally **prevented MCLS activation.** The number of cFOS-IR cells in this case (7.33 ± 2.73) was similar to aCSF/Sal group and significantly lower than the single administration of 150 nmol of ethanol (p=0.008) or NAC 120 mg/kg (p=0.005).

6. DISCUSSION



STUDY OF THE NEUROBIOLOGY OF ABSTINENCE AND RELAPSE TO ALCOHOL CONSUMPTION. EVALUATION OF N-ACETYLCYSTEINE AS A POTENTIAL ANTI-RELAPSE PHARMACOTHERAPY

Section adapted from:

Efficacy of N-acetylcysteine in the prevention of alcohol relapse-like drinking: Study in long-term ethanol-experienced male rats Cano-Cebrián, M. J., Fernández-Rodríguez, S., Hipolito, L., Granero, L., Polache, A., Zornoza, T. Published in *Journal of Neuroscience Research* 99.2 (2021): 638-648.

Different brain oxidative and neuroinflammation status in rats during prolonged abstinence depending on their ethanol relapse-like drinking behavior: Effects of ethanol reintroduction Fernández-Rodríguez, S., Cano-Cebrián, M. J., Rius, S., Pérez, S., Guerri, C., Granero, L., Zornoza, T., Polache, A. Published in *Drug and Alcohol* Dependence 232 (2022): 109284.

N-Acetylcysteine normalizes brain oxidative stress and neuroinflammation observed after protracted ethanol abstinence: a preclinical study in long term ethanol experienced male rats Fernández-Rodríguez, S., Cano-Cebrián, M. J., Esposito-Zapero, C., Pérez, S., Guerri, C., Zornoza, T., Polache, A. Published in *Psychopharmacology* (2023).

One of the most challenging problems in drug addiction therapy is the high rate of relapse in abstinent individuals. It is well established in the clinical setting that even after successful detoxification and abstinence treatment, an AUD patient remains at risk of relapse. Drug craving may even incubate over time, leading to a relapse risk several months after detoxification (Pickens et al., 2011). That is why **identifying the molecular mechanisms related to relapse in vulnerable individuals could help the development of more effective treatments** (Zhang et al., 2019). Recent reviews have highlighted the lack of papers studying the neurobiological alterations related to the different stages of the addiction cycle. Concretely, they have recognised the importance of shedding light on the alterations occurred during the abstinence period and, more precisely, during the protracted abstinence, since they could be related with the craving and drug-seeking behaviour that precedes relapse (Gipson et al., 2021; Namba et al., 2021).

As the study of the neurobiology of relapse behaviour is difficult to carry out in patients, the use of suitable animal models is necessary (Spanagel, 2017). Thus, we have designed the present research based on the application of the ADE preclinical model. This animal model allowed us to:

- Classify individuals depending on their vulnerability to display ethanol relapse behaviour.
- Conduct a comparative study between the different subpopulations in order to identify molecular differences and analyse whether they correlate with their vulnerability to relapse behaviour.
- Assess the preclinical efficacy of NAC as anti-relapse treatment for the vulnerable population.

— Study the potential mechanisms of action through which NAC is able to prevent ethanol relapse behaviour.

6.1. Using the ADE model for the identification of vulnerable population to relapse behaviour

Modelling AUD, which is a human mental disorder, in rodents is a challenge given its complexity. There is a large body of literature demonstrating that models of alcoholism in rats are an excellent basis for screening and developing pharmacological treatments for AUD (Belin-Rauscent et al., 2016; Bell et al., 2012). Among all the developed preclinical paradigms, **the ADE model** is probably one of the most commonly used preclinical approaches to study the ethanol relapse-like drinking behaviour due to its face, predictive and ecological validity (Bell et al., 2012; Spanagel, 2017). This preclinical model tries to include the entire range of the addiction cycle: drug taking, withdrawal and craving during periods of drug abstinence and relapse, which mimics human behaviour since nearly all AUD patients have undergone many relapse cycles throughout the course of their disease progression. This aspect supports the **face validity** of the model (Leong et al., 2018). The **predictive value** of the ADE preclinical model is well illustrated by the fact that two approved medications for alcohol relapse prevention —acamprosate and naltrexone— were developed by means of adequate animal models, more concretely, this particular ADE model, and then translated into a clinical setting (Spanagel, 2017). One of the minor limitations of this animal model could be that the ethanol abstinence and re-access are not freely controlled by the animal, hence it does not allow us to study the motivation to ethanol-seeking that

leads to relapse (Lê & Shaham, 2002). Even though new preclinical models that incorporate voluntary abstinence are being developed (abstinence is induced by introducing adverse consequences to drug taking or seeking, or by providing naturally exclusive choices between the self-administered drug and non-drug rewards). Nonetheless, their postdictive validity using approved medications remains unreported (Fredriksson et al., 2021).

Human alcohol consumers do not have a homogeneous behaviour: i.e., only a percentage of humans that consume drugs of abuse become addicted, which suggests that genetics and environmental factors are determining in the development of AUD (Goltseker et al., 2019). The use **of non-preferent animal strains** when studying vulnerability to ethanol relapse, allowed us to study this **heterogeneity.** The interindividual variability of our experimental subjects represents a critical strength of the current study, since this variability may provide valuable information about the neurobiological features that underlie addiction propensity (Kuhn et al., 2019). Conversely, a huge amount of preclinical research on alcoholism has been conducted using **selectively bred rat lines** (Bell et al., 2012). Thus, in this field, the scientific community has worked to develop rodent strains based on their high alcohol preference or high alcohol intake (Bell et al., 2006; Stewart & Li, 1997). The use of these rodent strains ensures higher blood ethanol levels and higher psychoactive effects. Nonetheless, the use of a biassed population would not facilitate the identification of different neurobiological aspects that could be associated with ethanol relapse vulnerability. In general, the use of preferent strains diminishes

data generalisability and the translational power of the obtained data (Spanagel, 2017).

On the one hand, in the present thesis, the use of two cohorts (male and female) of **non-selected Wistar rats** exposed to ethanol consumption under the **ADE model**, has demonstrated to be useful to identify and categorise two different subpopulations of male (see Figure 21 and Figure 25) and female (Figure 32 and Figure 36) rats that showed specific behavioural endophenotypes related to their **ethanol relapse-like drinking behaviour.** This finding was crucial to conduct a subsequent comparative study of the neurobiology of relapse.

On the other hand, the results of experiment 1 also suggested that the manifestation of the ethanol relapse-like drinking behaviour correlates with the pattern of voluntary ethanol intake displayed by the animals in both male and female rats. Specifically, male animals with a high probability of displaying ethanol relapse-like drinking behaviour (called “ADE group”) displayed lower **alcohol intake** rates than those observed in the no-ADE group, as can be appreciated in Figure 22. In the case of female rats (Figure 33), a similar trend was observed, i.e., ADE female rats displayed lower alcohol intake rates than no-ADE rats. However, the statistical analysis only detected a nearly significant effect when the subpopulation factor was analysed ($p=0.130$). Hence, this last observation should be cautiously considered in the case of our female rats.

Fredriksson and colleagues suggested that the high basal intake displayed by their rats may explain the lack of ADE observed due to a ceiling effect. They reported that, under their experimental conditions,

their male rats that consumed high levels of ethanol intake (around 4.7 mg/kg/day) did not display the ADE phenomenon. Consequently, they decided to exclude them when studying relapse (Fredriksson et al. 2015; 2023). The ethanol consumption rates of our male rats (ADE and no-ADE) are far from those reported. However, these data are not comparable with ours because these researchers used another rat strain (male Wistar-Han rat) and a different protocol (intermittent access to 20% ethanol), a protocol which usually induces high ethanol consumption rates in rodents (Carnicella et al., 2014; Simms et al. 2008).

Similar conclusions were obtained in the present thesis when **total ethanol preference,** instead of ethanol intake, was analysed. Firstly, **total ethanol preference was significantly increased after experiencing each deprivation period only in ADE rats.** This behaviour was observed in both male and female rats (Figure 25 and Figure 36 respectively). Curiously, only after the first deprivation period the preference for ethanol did not significantly augment in female ADE rats. However, it can be observed that the increase in ethanol intake after the first deprivation period is accompanied by an increase in total fluid intake (depicted in Figure 39). Thus, an increase in water consumption would explain this lack of change in the ethanol preference of ADE female rats in this particular deprivation period.

Secondly, total ethanol preference was clearly lower in the ADE than in the no-ADE population. This behaviour was clearly observed in both male (Figure 26) and female (Figure 37) rats. The above-mentioned differences were extremely evident when total ethanol preferences before each deprivation period were compared between groups (Figure

24 for males and Figure 35 for females). The existence of a ceiling effect could explain, at least in part, this behaviour.

Altogether, our results demonstrate that **animals more vulnerable to relapse display lower rates of ethanol intake and lower ethanol preference.** This result is consistent with previous observations from the study of the ADE manifestation in various alcohol-preferring rat lines, which are widely used in alcohol research (Bell et al., 2017). Concretely, after a single deprivation period, neither sP, nor AA lines showed an ADE phenomenon (Agabio et al., 2000; Sinclair & Tiihonen, 1988) and only alcohol-preferring P rats exhibit a robust ADE increasing their alcohol consumption approximately 2-fold on the first re-exposure day (McKinzie et al., 1998). A comparative study carried out by Vangeliene and collaborators reported that Wistar and P rats manifested ADE but HAD and AA rats did not. Thus, they concluded that these rats differentially responded to ethanol deprivation and stress, showing that their genetic background affected relapse-like drinking and stress-induced drinking (Vengeliene et al., 2003). It should be considered that these selective breeding rat lines emerged for the selection of animals characterised by their high ethanol preference or excessive ethanol drinking, and not on the strength of their relapse-drinking behaviour, fitting with our present results.

Finally, the ethanol preference for each dilution was also analysed and a great difference in the ethanol consumption pattern between ADE and no-ADE animals was again detected. In particular, in the case of male rats (Figure 27 and Figure 28), the ADE subpopulation displayed lower preference for 5% ethanol solution than no-ADE group and leant towards the consumption of ethanol from the 20% bottle. The

preference for more highly concentrated ethanol may be explained by the rat motivation to rapidly increase the blood ethanol concentrations, which reflects a more **compulsive ethanol drinking behaviour** (Foo et al., 2022; Vengeliene et al., 2014). Similarly, in the case of female rats (Figure 38), no-ADE animals displayed a higher preference for 5% ethanol dilution than ADE animals whereas the ADE group showed higher preference for 10% and 20% ethanol dilutions than the no-ADE group. Strikingly, in the case of our female rats, after 6 months of ethanol exposure, the differences in ethanol preference between both groups were vanishing. Concretely, the preference of the ADE group leant towards those displayed by no-ADE animals. Apparently, if the preference for more concentrated dilutions of ethanol reflects the motivation to increase the blood ethanol concentration (Vengeliene et al., 2014), the obtained results suggest that our ADE female animals seemed to be losing their compulsive ethanol drinking behaviour by the end of the experiment.

Alcohol accounts for approximately 5% of all deaths in men, while only accounting for 1% of all deaths in women and, while men are often diagnosed with more alcohol use issues than women, this gender gap is slowly closing (Henricks et al., 2016). Hence, another aspect worth mentioning from our experiments is the difference in the alcohol intake rate displayed by male and female rats. We calculated the average ethanol intake from the three basal and reintroduction periods and compared them through unpaired Student's t-tests between male and female rats for both subpopulations (ADE and no-ADE). According to our data (see Table 13), female rats (ADE and no-ADE) displayed higher

ethanol intake than male rats not only during the basal days but also during the alcohol re-access period.

	Alcohol intake (ADE subpopulations)		Alcohol intake (no-ADE subpopulations)	
Sex	Basal	Reintroduction	Basal	Reintroduction
Males	1.99±0.11	2.89±0.14	2.96±0.26	3.06±0.30
Females	3.43±0.29	4.97±0.31	4.50±0.29	4.66±0.30
Significance	p<0.0001	p<0.0001	p=0.0007	p=0.0009

Table 13. Comparison of ***ethanol consumption*** *(expressed as g/kg/day) between male and female rats for both ADE and no-ADE subpopulations. The average value of the three basal or reintroduction periods is calculated to be compared between male and female rats. The results of unpaired Student's t-test are shown in the bottom row.*

Then, we used the same method to compare the ethanol preference displayed by both cohorts of animals and we found no difference in any case between their ethanol preference (Table 14).

	Alcohol preference (ADE subpopulations)		Alcohol preference (no-ADE subpopulations)	
Sex	Basal	Reintroduction	Basal	Reintroduction
Males	44.02±2.95	60.32±3.10	68.93±4.78	69.46±3.94
Females	49.98±4.78	63.06±3.51	70.19±5.72	72.36±5.58
Significance	p=0.271	p=0.564	p=0.871	p=0.689

*Table 14. Comparison of **ethanol** preference (expressed as % of total fluid intake) between male and female rats for both ADE and no-ADE subpopulations. The average value of the three basal or reintroduction periods is calculated to be compared between male and female rats. The results of unpaired Student's t-test are shown in the bottom row.*

According to these comparisons, under our experimental conditions, the ethanol intake clearly reflects a difference in the alcohol drinking behaviour displayed by male and female individuals. A recent study has also compared the differences between males and females in their ethanol intake and preference. This group used a different rat strain (Long-Evans) and a different preclinical model (20% ethanol intermittent access) than ours. They found that female rats normally drank twice as much as male rats and displayed higher preference for ethanol consumption than males (Pirino et al., 2022).

In summary, the present results indicate that the applied criteria to categorise male rats clearly allowed us to distinguish two subpopulations: ADE and no-ADE. They **differ in their ethanol relapse-like drinking behaviour** but also, ADE rats typically displayed lower ethanol intake and lower total ethanol preference, but a higher preference for 20% ethanol dilution than no-ADE rats. These phenotypic differences between ADE and no-ADE subpopulations were also detected in the cohort of our female Wistar rats. However, it is remarkable that, at the end of the experiment conducted in female rats, the preference differences were progressively vanishing. Besides, from our experiments, 70% of male animals were assigned to the ADE group, while only 58% of female rats met the criteria of ADE animals. These results suggest that, under our experimental conditions, the

dissimilarity between both subpopulations is not as intense in females as it is in males. Consequently, it was considered that the cohort of male Wistar rats was more suitable to **identify the potential pathways involved in the pathophysiology of ethanol relapse.** However, the manifestation of ADE phenomenon at the third deprivation period was robust for male and female ADE subpopulations (Figure 21 and Figure 32), therefore, both groups of ADE animals (male and female) were considered appropriate to conduct a **pharmacological experiment to evaluate the potential anti-relapse efficacy of NAC.**

6.2 Exploring the neurobiology of the disease in different phases of AUD

A plethora of research have been performed during the last decades focused on the neuroadaptations that underlie the development of AUD (Alhaddad et al., 2014; Baxter-Potter et al., 2017; Healey et al., 2021; Schneider et al., 2017). However, few of the published papers are focused on the alterations that occur during the alcohol abstinence and reintroduction periods. This information is crucial to understand the progression of the disease. Besides, most of the published studies that explore the neurobiological changes caused by chronic ethanol exposure show some limitations as they have been performed using: (i) non-voluntary ethanol consumption protocols or (ii) alcohol-preferent rat strains. Thus, one of our aims was to study how certain changes caused by alcohol misuse evolve during the progression of the disease. To do so, we designed a three-stage study to explore oxidative stress and several neuroinflammatory markers after:

- **Chronic voluntary ethanol intake.**

- Abstinence period, more specifically, after a **prolonged abstinence period** (21 days), which probably leads to the relapse phenomenon.

- **24 hours of alcohol reintroduction** after the experience of a long-term abstinence period.

Once the rats were separated in two clearly differentiated categories according to their relapse-like drinking behaviour (ADE and no-ADE) in experiment 1, the male cohort was used **to differentially study and**

compare several neurobiological alterations that could lead to the relapse process.

6.2.1 Brain oxidative stress and neuroinflammation may be associated with vulnerability to relapse

It is widely known that the central nervous system is highly sensitive to oxidative stress, because of its high oxygen consumption and lipid content as well as its low antioxidant defence (Halliwell, 2006). Furthermore, the literature strongly demonstrates, in the case of drug addiction, the existence of an oxidative stress–neuroinflammation vicious cycle perpetuating both oxidative stress and neuroinflammation (Berríos-Cárcamo et al., 2020). Thus, in experiment 3 we explored how (i) chronic ethanol exposure, (ii) long-term abstinence and (iii) relapse to ethanol consumption affects the brain redox and neuroinflammatory status. Finally, as we had two different subpopulations (ADE and no-ADE) we could hypothesise that the potential alterations determined during the abstinence period could be associated with the vulnerability to ethanol relapse-like behaviour. The most relevant results of experiment 3.1 and experiment 3.2 are collected in Figure 60 and discussed in the following sections.

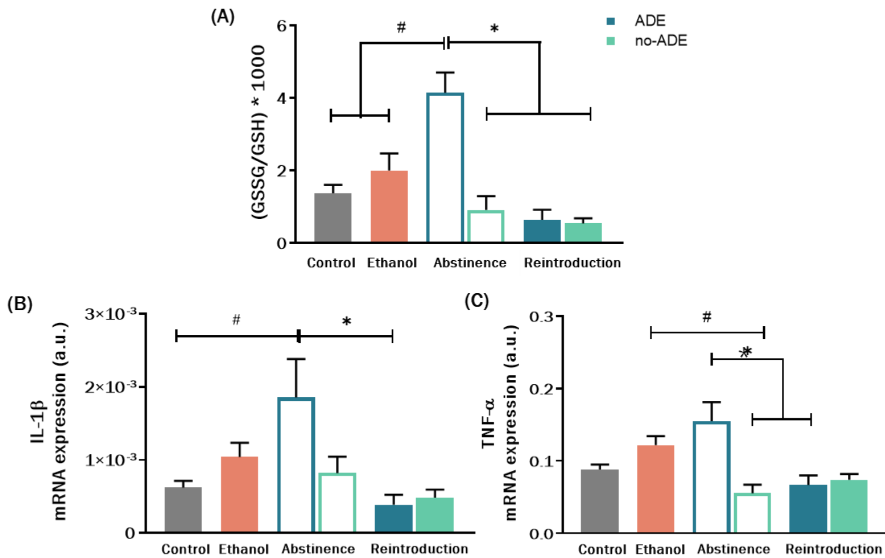


Figure 60. (A) ***oxidative stress*** *status (determined in HIP) and the mRNA levels of (B)* ***IL-1β*** *and (C)* ***TNF-α*** *(determined in PFC) at the three stages of the addiction process: continuous ethanol consumption, prolonged abstinence and ethanol reintroduction. The depicted experimental conditions are: animals chronically exposed to water consumption (control) in solid grey; animals chronically exposed to ethanol consumption (ethanol) in solid red. The results from ADE animals are shown in blue bars and no-ADE in green bars. For these two populations, framed bars represent the abstinence condition (determined after 21 days of ethanol withdrawal) while solid bars represent 24 hours of ethanol reintroduction. Data are represented as mean ± SEM [* $p<0.05$; # $p<0.05$]*

We decided to explore the redox status within HIP and AMG given that AMG is considered one possible locus for alcohol withdrawal-anxiety, a process which is related to craving and relapse behaviours (Harper et al., 2019). On the other hand, a great deal of evidence has demonstrated that ethanol induces oxidative damage in HIP (Almansa et al., 2013; Ezquer et al., 2019; Johnsen-Soriano et al., 2007; Quintanilla et al., 2018; Reddy et al., 2013; Scolaro et al., 2012).

Besides, we analysed the mRNA expression levels of several neuroinflammatory mediators in PFC, since it is highly affected by ethanol exposure (Abernathy et al., 2010) and many studies of this sort have been carried out in this area (Knapp et al., 2016; Sanchez-Alavez et al., 2019; Schneider et al., 2017). It would have been ideal to study both oxidative and inflammatory processes in the same brain areas. Unfortunately, the amount of tissue available was not enough to perform qPCR and mass spectrometry experiments in the selected nuclei.

VOLUNTARY ETHANOL INTAKE DOES NOT ALTER OXIDATIVE STATUS AND INFLAMMATORY PROCESSES IN THE BRAIN

A great deal of evidence about chronic ethanol exposure and altered oxidative status can be found in the literature (Akhtar et al., 2017; Fernandes et al., 2017; Ramachandran et al., 2003; Uys et al., 2014). Strikingly, the results obtained in the present thesis revealed that, under our experimental conditions, **GSH and GSSG levels were not modified by prolonged chronic ethanol consumption** neither in the HIP nor AMG (Figure 44 and Figure 45). It should be taken into account that in the papers that reported positive effects: (i) rats were exposed to the Lieber-deCarli alcohol liquid diet, which augments the rate of ethanol intake by the animal (Almansa et al., 2013; Johnsen-Soriano et al., 2007), (ii) rats received high ethanol doses (3 g/kg) through the intraperitoneal route (Scolaro et al., 2012), or (iii) experiments were performed using selected rat strains that drank around 12 g/kg/day (Ezquer et al., 2019). Conversely, in other reports on which lower ethanol doses were assayed, for example by oral gavage (1.5 g/kg), no changes were observed i.e., GSH remained unaltered in the frontal

cortex, HIP or STM of male Sprague–Dawley rats (Sommavilla et al., 2012). Hence, our results are in accordance with the literature given that, in our experimental conditions, male Wistar rats displayed a voluntary average ethanol intake of **2.21 ± 0.12 g/kg per day**, which is a low ethanol dose compared to previous data.

Similar conclusions can be achieved when we analysed the mRNA expression of seven neuroinflammatory mediators (IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB and NLRP3) in PFC (Figure 46 and Figure 47). Under our experimental conditions, **long-term chronic ethanol consumption did not alter any inflammatory mediator in PFC.** Once again, this result may be a consequence of the lower ethanol consumption rate displayed by our animals, since different studies conducted with male and female rats have shown that chronic ethanol exposure augments cytokine expression in HIP and cortex. In fact, in these studies, animals were exposed to high ethanol levels through ethanol administration following a forced schedule (10 g/kg by oral gavage for 10 weeks) (Tiwari et al., 2009), or using the Lieber-deCarli diet during 5 months (Vallés et al., 2004). It has also been reported that high rates of ethanol consumption caused astrocytosis, (marker of neuroinflammation) in HIP of female UChB rats (Ezquer et al., 2019). Qin and collaborators also reported that male C57BL/6J mice intragastrically treated with 5 g/kg/day for 10 days showed increased brain levels of TNF- α and MCP-1 (Qin et al., 2008).

All in all, our experimental substrate, a wild-type non-selected Wistar rat, under a more ecological model of voluntary ethanol consumption, showed **moderate alcohol consumption rates that were not enough to**

cause a redox imbalance in HIP and AMG or trigger inflammatory processes in PFC.

PROLONGED ETHANOL ABSTINENCE INDUCE OXIDATIVE DAMAGE AND INFLAMMATION ONLY IN ANIMALS THAT DISPLAY ETHANOL RELAPSE-LIKE DRINKING BEHAVIOUR

The study of the alterations occurred during the long-term abstinence period provided interesting results. Concretely, during a protracted abstinence, a **correlation between hippocampal oxidative stress and cortical inflammatory response with the propensity to display ethanol relapse-like drinking behaviour** was detected (Figure 60).

Regarding the effects associated with prolonged ethanol abstinence on brain redox status, our results showed that after three weeks of ethanol abstinence, a huge difference in hippocampal oxidative stress levels between the ADE and no-ADE subpopulations was detected. In particular, the GSSG/GSH ratio was drastically higher in the ADE group than in the no-ADE subpopulation (Figure 44 C and Figure 60). This difference was exclusively detected in the HIP as the data obtained in the AMG revealed no changes in the redox status (Figure 45). To the best of our knowledge, this relation between oxidative stress and vulnerability to relapse has never been described before. Moreover, the redox imbalance detected during protracted abstinence has only been reported once in the literature. Specifically, Jung and collaborators found increased levels of oxidative stress markers in HIP and cerebellum of Sprague-Dawley rats after three weeks of abstinence, when they were analysing sex differences in the alterations caused by ethanol withdrawal. However, these results were obtained using a

model of forced alcohol intake that increases the risk of brain damage. Interestingly, they found a less severe oxidative damage in ethanol withdrawn female rats than in male rats (Jung & Metzger, 2016). The lack of studies focused on the oxidative alterations during long-term abstinence is evinced in a recent review which claims that it is still unknown if the alterations in oxidative brain status persist after the initial period of ethanol abstinence (Clergue-Duval et al., 2022). In the literature there is evidence of the increased oxidative stress status during early abstinence in male Wistar rats at systemic (Gonzaga et al., 2015) and hippocampal levels (Elibol-Can et al., 2011). In fact, Elibol-Can and colleagues suggested that ethanol withdrawal may cause equal to or even more severe brain damage than the ethanol itself. Even so, other preclinical findings suggest that the redox imbalance during abstinence is temporary: a study showed that oxidative stress in HIP tends to be reduced as time goes by during abstinence in female Sprague-Dawley animals (Jung et al., 2004). In the clinical setting, Huang and colleagues reported elevated levels of serum oxidative stress markers after 1–2 weeks of alcohol detoxification in patients (Huang et al., 2009). In any case, these outcomes did not necessarily mean that the alterations in redox status during early abstinence are not related with ethanol relapse in the long term, as oxidative stress causes neuronal damage (Jung & Metzger, 2010) and enhances protein S-Glutathionylation (Womersley & Uys, 2016) which may have an impact on the subsequent relapse.

Likewise, data derived from the study of the neuroinflammatory status during prolonged abstinence was parallel to the redox analysis: significant differences were found between the ADE and no-ADE

groups. Specifically, **the mRNA expression of the IL-1β was enhanced after 21 days of ethanol abstinence only in the ADE population. Moreover, the levels of TNF-α were significantly higher in the ADE group in comparison with the no-ADE subpopulation during abstinence** (Figure 46 A and Figure 47 A, respectively). The active neuroinflammatory process during ethanol withdrawal has already been reported during short-term abstinence in preclinical studies. For instance, male Wistar rats exposed to 2 g/kg alcohol twice a day by oral gavage for 30 days, that underwent a 5-days withdrawal period showed increased values of TNF-α, IL-1β, IL-6 and IL-18 in the HIP and the frontal cortex (Schneider et al., 2017). Similarly, increased cytokine levels (IL-1β and CCL-2) were found in cortex and HIP of rats that had received a continuous 7% (w/v) ethanol diet followed by a 29-hour withdrawal period (Knapp et al., 2016). When the withdrawal period analysed is extended results are less consistent: the increase in brain MCP-1 after 10 days of ethanol treatment remained for at least 7 days of ethanol abstinence in mice, whereas the increase in TNF-α expression caused by ethanol exposure was restored during the abstinence period (Qin et al., 2008). Other authors reported that 7 days were sufficient to revert the increase in brain CCL2, TNF-α and IL-1β previously detected in the cortex of male Sprague-Dawley rats chronically exposed to ethanol consumption (Whitman et al., 2013). When neuroinflammation was analysed after a long-term abstinence, one study found increased levels of TNFα and MCP-1 in the frontal cortex of adolescent male Wistar rats 25 days after intermittent alcohol exposure (Vetreno et al., 2013). Conversely, other authors showed that neither 1 nor 28 days of ethanol withdrawal did affect the levels of several cytokines (including TNF-α and IL-1β) in the frontal cortex of

male Wistar rats, although it did increase the expression of the microglial marker Iba-1 (Sanchez-Alavez et al., 2019). This concrete study demonstrates the importance of paying attention to microglia: although the cytokine levels were unaltered, the microglial marker was overexpressed. To illustrate, a recent paper indicates that the high magnification study of microglial morphology revealed that chronic intermittent ethanol exposure caused structural changes similar to those occurred during Alzheimer's disease or ageing (Siemsen et al., 2021). Thus, a future set of experiments to explore how protracted abstinence activates or not microglial cells in the ADE and no-ADE groups would enrich our research. In the clinical setting, Yen and collaborators reported that AUD patients had higher plasma cytokine levels than healthy controls during early withdrawal. Nonetheless, the levels of cytokine expression were falling over time in abstinent patients (Yen et al., 2017). Contrarily, other authors reported increased circulating levels of TNFα and IL-6 after three weeks of abstinence (Leclercq et al., 2012). However, it must be taken into account that liver inflammation may be responsible, at least in part, of these circulating levels of cytokines.

Even though the discrepancy around the reported data, some of the mentioned studies reveal that oxidative stress markers and inflammatory mediators during abstinence do not remain altered forever, tending to evolve in the course of time. Hence, the analysis of the oxidative and inflammatory status at different moments during the abstinence period (beyond 21 days) will help us to better understand the dynamics that occurs during this stage. In any case, our experimental procedure (to separate abstinent animals in two

subgroups based on its vulnerability to display ethanol relapse-like drinking behaviour) allowed us to link the disbalances in the oxidative status and inflammatory mediators with the vulnerability to relapse.

All in all, our outcomes suggest that in a brain region-dependent way, oxidative stress and the proinflammatory signalling might play a **critical role in triggering alcohol craving and relapse**. This result largely supports **the hypothesis of a proinflammatory status linked to increased risk of AUD** (Warden et al., 2016).

6.2.2 The alcohol reintroduction seems to reset the oxidative and the neuroinflammatory disbalance triggered by abstinence

Notwithstanding ethanol is widely known to be a prooxidant substance (Wu & Cederbaum, 2003), the effect of 24 hours of ethanol reintroduction in brain redox status, under our experimental conditions, was really astonishing. As can be observed in Figure 60, the reintroduction to ethanol consumption rapidly alleviated the oxidative stress detected in the HIP during the abstinence period in the ADE rats. The effects of ethanol re-access depended on the different endophenotypes displayed by the rats. In the same line, the effects of 24 hours of ethanol reintroduction strongly diminished the levels of IL-1β and TNF-α only in the ADE rats (Figure 60). Conversely, Cuitavi and colleagues who exposed female Sprague-Dawley rats to an ethanol intermittent access model, reported that the levels of IL-1β in PFC were significantly lower during protracted abstinence than after 5 sessions of alcohol reintroduction. However, these experiments were performed in animals that did not manifest an ethanol relapse-like drinking

behaviour after the abstinence period and their experimental schedule is not comparable to ours (Cuitavi et al., 2021).

Apparently, it seems that the **short ethanol re-access had an alleviating effect that counteracted the abstinence-induced oxidative stress and proinflammatory condition.** In our opinion, these effects could be considered "paradoxical". In any case, they allowed us to study the mechanism of action of NAC in ethanol relapse in absence of ethanol, as its presence potentially could interfere in the altered neuroimmune and oxidative status and would mask NAC effects. On the other hand, having observed this result made us wonder if this effect is transitory. To answer this question satisfactorily, the effects of ethanol reintroduction on oxidative stress and neuroinflammation at different time points, as well as the plausible underlying mechanisms should be an avenue of future investigation.

6.2.3 Effect of chronic ethanol intake and prolonged abstinence on the glutamate transporters expression

Since the glutamate hypothesis of addiction was proposed (nearly two decades ago), the literature focused on the exploration of the relationship between the glutamatergic neurotransmission and the different stages of the addiction process is constantly growing. It has been claimed that an impaired glutamatergic neurotransmission is related to drug intake, craving and relapse (Reissner & Kalivas, 2010; Scofield & Kalivas, 2014). Particularly, using preclinical models, many researchers have put huge efforts into studying how the consumption of different drugs of abuse affects GLT1 and xCT expression within the MCLS since they could be potential therapeutic targets.

On the one hand, it is widely known that withdrawal after prolonged periods of ethanol exposure increases glutamate levels within the nucleus accumbens (Rossetti & Carboni, 1995). Nonetheless, the results found in the literature about the effects of alcohol on GLT1- and xCT expression are not fully coincident. The high variability and discrepancies reported are probably related to different variables such as rat strains, the use of ethanol preferent vs naïve animals and, particularly, the ethanol administration paradigm used (Abulseoud et al., 2014). Once again, studies focused on the abstinence period, and specially protracted abstinence, are scant. That is why in experiment 4 we aimed to evaluate whether the expression of GLT1, GLAST and xCT in the STM, specifically in the nucleus accumbens and DS, is affected by chronic alcohol consumption or ethanol protracted abstinence. Due to the limited amount of available tissue, only the ADE population could be analysed during the abstinence in this experiment. The most interesting results were obtained in the nucleus accumbens. These results are depicted in Figure 61.

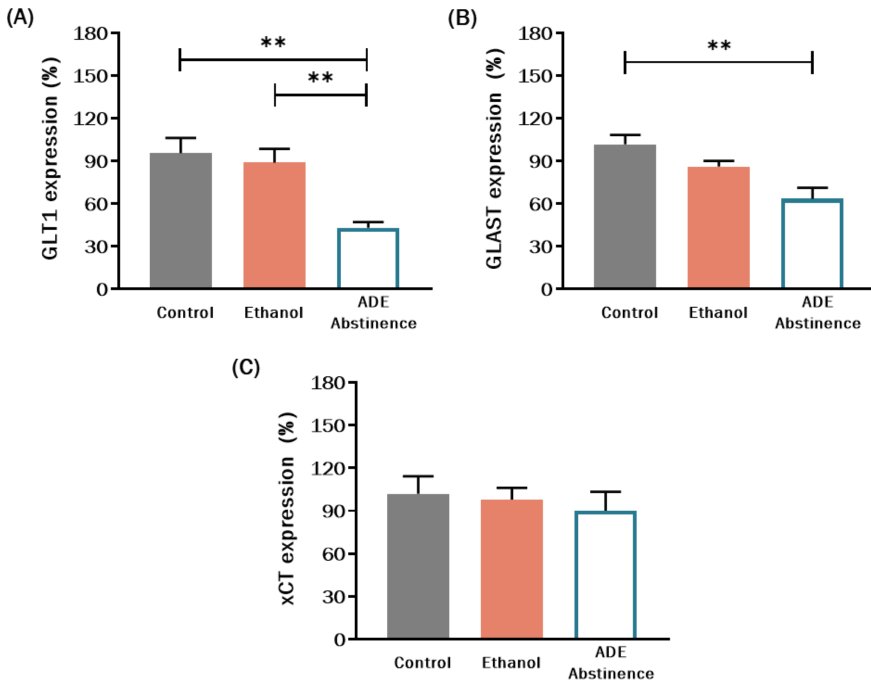


Figure 61. Effect of chronic ethanol exposure (red bars) and protracted ethanol abstinence for the ADE group (blue framed bar) on the expression of ***GLT1*** *(A),* ***GLAST*** *(B) and* ***xCT*** *(C) in the nucleus accumbens of male Wistar rats. Data are represented as mean ± SEM [** p<0.01]*

By and large, our data revealed that, under our experimental conditions, **chronic alcohol consumption did not alter the protein levels of GLT1, GLAST and xCT either in the nucleus accumbens or in the DS** (Figure 52 and Figure 53, respectively). Previous studies focused on their expression in **nucleus accumbens** showed that intermittent access to alcohol (IAA) seem to lead to different adaptations in GLT1 and xCT when compared to continuous access to ethanol (see Table 1 in the introduction section). Thus, in male P rats with continuous access to ethanol in their home cage, a downregulation of GLT1 expression has

been consistently reported (Alhaddad et al. 2014; Das et al. 2015; Hakami et al., 2016; Sari et al. 2013). However, no changes in GLT1 expression were observed when experiments were conducted in female rats (Ding et al., 2013; Ezquer et al., 2019), when an IAA paradigm was experienced (Pati et al., 2016; Stennett et al., 2017) or during a re-access to ethanol consumption after an abstinence period (Das et al., 2022; Ezquer et al., 2022). In another study, a GLT1 downregulation was detected in nucleus accumbens shell but not in core (Hammad et al., 2021). Similar results were obtained in the case of xCT levels (Das et al. 2022; Ding et al. 2013; Ezquer et al. 2019, 2022; Hakami et al., 2016; Hammad et al., 2021; Pati et al. 2016; Stennett et al. 2017). Concerning GLAST expression, no changes were observed in male P or female UChB rats that have continuous access to ethanol (Alhaddad et al., 2014; Ezquer et al., 2019; Hakami et al., 2016) while reduced expression levels were reported when female P rats were used (Ding et al., 2013). Overall, an in-depth analysis of the above data shows that **chronic ethanol intake provokes a decrease or no alteration of expression levels of these glutamatergic proteins.** Hence, our results are in accordance with those showing no modification in GLT1, GLAST and xCT levels in the nucleus accumbens of male rats after chronic alcohol exposure. Regarding **DS**, it is an area that has been explored to a lesser extent. However, we decided to study it since it is involved not only in the progressive development of compulsive drug seeking but also it has a key role in the loss of control over drug intake (Belin & Everitt, 2008; Vollstädt-Klein et al., 2010). Moreover, Ducret et al reported that cocaine intake induced a decrease in GLT1 expression in this area and NAC was able to reverse it (Ducret et al., 2016). According to our results, chronic ethanol consumption did not cause any changes

in the expression levels of any of the assayed glutamate transporters in comparison to the control group in this area (Figure 52 and Figure 53). These results are partially in accordance with the scarce data found in the literature. Thus, Morais-Silva et al. demonstrated no changes in xCT expression in the caudate area of mice which had received 2g/kg/day ethanol i.p. injections for 13 days (Morais-Silva et al., 2016).

The most remarkable outcomes in this experiment concerned the abstinence period in nucleus accumbens (Figure 61). **Animals exposed to long-term abstinence showed reduced expression of GLT1 and GLAST in nucleus accumbens.** In particular, a significant reduction in GLT1 levels during a protracted abstinence was found (around 45 %) with respect to the control and ethanol groups. In the case of GLAST expression, a significant decrease was also detected as a consequence of a protracted abstinence, showing a similar trend to that observed in GLT1. However, no changes in xCT levels were detected under any experimental condition (Figure 52). Unfortunately, the amount of biological sample was not enough to perform more experiments and analyse if this effect was exclusive of ADE subpopulation or if the abstinence period would also alter the expression of glutamate uptake proteins in the no-ADE group. Hence, we could not correlate this alteration with the manifestation of the relapse-like drinking behaviour. The literature focused on glutamate homeostasis in nucleus accumbens during the withdrawal period provide controversial results. For instance, studies performed with male rats reported that early abstinence conditions did not alter GLT1 and GLAST expression (Lebourgeois et al., 2019; Melendez et al., 2005), but downregulated

xCT expression (Lebourgeois et al., 2019; Peana et al., 2014). The only study found analysing the consequences of a 2-week abstinence period, showed no alteration in GLT1 and xCT protein expression, while GLAST levels were found to be diminished. However, these results are not fully comparable with ours, given that they were obtained in female P rats (Ding et al., 2013). With regard to the obtained results in DS during abstinence (Figure 53), our results contrast with those obtained by Abulseoud and collaborators who found a decrease in GLT1 in total STM after seven days of withdrawal. This result was obtained in male P and Wistar rats (Abulseoud et al., 2014).

Taken together, our results provide the first evidence of **how long-term voluntary ethanol drinking with repeated deprivation periods affects the expression of GLT1, GLAST and xCT proteins during long-term abstinence.** In summary, we observed a decrease in some of the glutamate uptake protein levels in nucleus accumbens that concur with the largest concentration of synaptic glutamate levels reported during prolonged abstinence in this brain area (Griffin et al., 2015; Pati et al., 2016). Moreover, our data, obtained with a preclinical model of voluntary ethanol intake and repeated abstinence, offers a more complete view of how repeated ethanol abstinence could be affecting glutamate transmission in AUD patients.

6.2.4 Highlights

The bottom line of this section is that there is an **altered brain oxidative and neuroinflammatory status after a three-week ethanol abstinence** in male Wistar rats previously exposed to a long-term ethanol experience. It was interesting to confirm that these changes only

occurred in animals that displayed ethanol relapse-like drinking behaviour. Moreover, the access to ethanol consumption alleviated them. Hence, targeting neuroinflammation and reducing oxidative stress could be a promising therapeutic strategy for individuals with AUD. Additionally, **a downregulation of glutamate uptake proteins GLT1 and GLAST in nucleus accumbens after protracted abstinence was detected.** Thus, further experiments are needed to elucidate if this condition is also related to the vulnerability to relapse.

We are aware that our research must be complemented with studies in a **female cohort of rats** to be translational to all the population, men and women. Specially, since it has been proposed that steroidal hormones have a crucial role in the immune functions involved in addiction (Gipson et al., 2021). Even if our first idea was to include these experiments in the present thesis, the vanishing differences between the female ADE and no-ADE groups in experiment 1.B persuade us to perform these experiments.

6.3 N-ACETYLCYSTEINE PREVENTS ALCOHOL RELAPSE-LIKE DRINKING BEHAVIOUR

The underlying mechanisms involved in the different phases of AUD development are not only multiple but also complex. Although a wealth of studies has highlighted the role of glutamate homeostasis in relapse in the last decades (Kalivas, 2009), research has started to identify alterations in oxidative stress and neuroimmune signalling associated with addiction-related behaviours, including relapse (Berríos-Cárcamo et al. 2020; Gipson et al. 2021; Knapp et al. 2016; Namba et al. 2021; Tapia-Rojas et al. 2018; Womersley et al. 2019). These observations have led to suggest several biological molecules and chemical agents to be used as novel pharmacotherapies to reduce drug consumption as well as prevent relapse. For this purpose, one of the most proposed compounds is **NAC** (Berríos-Cárcamo et al., 2020; Gipson, 2016; Namba et al., 2021). Accumulating evidence suggests that NAC may be effective in preventing relapse to drug use (Duailibi et al., 2017; Ducret et al., 2016). Nonetheless, preclinical and clinical reported outcomes are not clear on its efficacy, especially when the duration of the therapy is brief or the patient is not abstinent (LaRowe et al., 2013; Powell et al., 2019). Hence, we proposed to use the group of animals identified as more vulnerable to relapse in experiment 1 (the ADE group) to:

-Explore the **efficacy of NAC to prevent ethanol relapse-like drinking behaviour** in a cohort of animals with a long-term ethanol exposure that have experienced several episodes of abstinence under the ADE model (experiment 2)

-Explore the **mechanism of action** underlying the NAC anti-relapse effect (experiment 4).

6.3.1 Pharmacological studies to assess NAC efficacy

Despite preclinical and clinical research has increasingly explored the potential efficacy of NAC to regulate/modify drug-related behaviours (McClure et al., 2014), the literature is mixed on its efficacy, particularly depending on the drug of abuse assayed:

- NAC has been found to reduce **cocaine**-seeking behaviour in rats (Amen et al., 2011; Baker et al., 2003; Kupchik et al., 2012; Murray et al., 2012), to prevent the escalation of drug intake (Madayag et al., 2007), and to facilitate self-imposed abstinence after cocaine escalation (Ducret et al., 2016).

- Preliminary clinical data indicate that repeated administration of NAC to **cocaine-dependent human subjects** produced a significant reduction in craving (Amen et al., 2011). The clinical trial conducted by LaRowe and colleagues failed to demonstrate that NAC reduces cocaine use in actively using cocaine-dependent individuals, but they demonstrated its anti-relapse effect in patients who had already achieved abstinence from this drug (LaRowe et al., 2013). Besides, a recent inpatient study provided the first clinical evidence that NAC can suppress cocaine-primed and cocaine-seeking behaviour (Woodcock et al., 2021).

- **Nicotine** self-administration, extinction response, seeking behaviour and cue-induced reinstatement have also been reduced in rats treated with NAC (Moro et al., 2018, 2020; Powell et al., 2019; Quintanilla et al., 2018; Ramirez-Niño et al., 2013).

- **Human smokers** treated with NAC reported a reduction in cigarette smoking but without any effect on the measurements of withdrawal and craving symptoms (Knackstedt et al., 2009). Nevertheless, Froeliger and colleagues reported that NAC was able to prevent nicotine craving (Froeliger et al., 2015).

- In a preclinical setting, effects of NAC on **heroin**-seeking behaviour have also been reported (Hodebourg et al., 2019; Zhou & Kalivas, 2008), and a potential role in the management of **methamphetamine** dependence has also been claimed (McKetin et al., 2017).

Studies with **ethanol** evaluating the preclinical effectiveness of NAC (discussed below) are scarce. Moreover, the preclinical studies in which NAC has been suggested to prevent relapse, have relied upon a single period of abstinence (Lebourgeois et al., 2018; Quintanilla et al., 2018) which does not mimic the clinical condition, as most patients looking for treatment have experienced multiple cycles of abstinence and relapse. Hence, the use of the ADE preclinical model in the present thesis tried to overcome this limitation.

An intermittent administration of NAC, for 14 days, was selected to conduct this experiment. Concretely, we administered NAC once a day through a subcutaneous injection protocol, previously validated by

other authors (Corbit et al., 2014; Ducret et al., 2016; Quintanilla et al., 2016). NAC treatment was assayed in a cohort of male (experiment 2.A) and female (experiment 2.B) rats that had previously been selected based on their elevated vulnerability to display ethanol relapse-like drinking behaviour (ADE subpopulations). This study has been conducted in male and female rats since sex differences in the psycho-pharmacological effects of NAC have been previously reported (Goenaga et al. 2020; Monte et al. 2020)

According to the results obtained in experiment 2.A (performed in males), NAC prevented the ADE manifestation at the two administered doses NAC 60 and 100 mg/kg (Figure 40). Interestingly, our data reflect an **inverted U-shaped dose-effect curve** since the anti-relapse effect of NAC was higher with 60 mg/kg/day than 100 mg/kg/day. The administration of NAC 60 mg/kg significantly reduced the amount of ethanol consumed after the abstinence period whereas the NAC 100 mg/kg dose counteracted the increase in ethanol intake that characterises the manifestation of the ADE phenomenon. This result could agree with the data reported by Kupchick and collaborators in 2012. They found, using electrophysiological techniques and under *in vitro* conditions, that NAC could activate or inhibit the glutamatergic transmission in nucleus accumbens depending on the administered dose. Specifically, low concentrations of NAC inhibit glutamate transmission onto nucleus accumbens core spiny cells through the activation of presynaptic mGluR2/3. Nonetheless, higher concentrations of NAC counteract this effect by also stimulating mGluR5 at the postsynaptic level. After these observations, they concluded that the effect of NAC on relapse to cocaine seeking

depends on the balance between stimulating mGluR2/3 and mGluR5 in this brain region (Kupchik et al., 2012). Although our preclinical conditions are far from those used in that procedure, the results obtained point in the same direction and underline the importance of **selecting the appropriate dose of NAC.** This result set in motion the design of experiment 5.A of the present thesis, in which this hypothesis was explored, but under *in* vivo conditions.

The **total liquid consumption** was also evaluated in order to rule out that a possible loss of interest in the consumption of liquids could have caused the observed reduction in ethanol intake displayed by the animals treated with NAC. As depicted in Figure 41 A, after receiving NAC treatment, total liquid consumption was not significantly reduced when compared with basal values in any of the experimental groups, suggesting that NAC could have a specific effect on the mechanisms that mediate ethanol relapse. Curiously, **total fluid intake was significantly increased, but only in the group of animals treated with NAC 100 mg/kg.** In our opinion, the heterogeneity of the population could explain this result. Indeed, after animals were randomly assigned to different experimental groups, we determined no group differences not only in baseline ethanol consumption but also in total fluid intake. Only after we detected this increase in the total liquid consumption of the NAC 100 mg/kg group, was the basal fluid intake analysed and the results revealed the existence of significant differences between groups [$F(2, 117) = 5.556$; $p = 0.005$]. According to this result, we suspect that animals coincidentally assigned to 100 mg/kg NAC displayed a lower total fluid intake than the ones assigned to other groups, providing a plausible explanation for the obtained result.

Although this outcome was quite intriguing, in our opinion, it did not invalidate the obtained outcomes. Regarding animals' **weight**, it was not altered by the treatment in any experimental group.

Next, we tried to explore the anti-relapse effect of NAC in a cohort of female rats. First, according to the experiment 1.B, only 22 of 38 female rats met the criteria to be assigned to the ADE subpopulation. Consequently, in experiment 2.B, we could only assay the anti-relapse effect of one dose of NAC. The assayed dose, 60 mg/kg, was chosen for being the more effective in our previous experiment. According to our data, **a significant reduction in ethanol intake was detected in female Wistar rats after being treated with NAC 60 mg/kg** (Figure 42). Unfortunately, the obtained results in experiment 2.B cannot be considered completely successful, given that **animals treated with vehicle did not manifest the ADE phenomenon.** Thus, we cannot confirm that NAC treatment prevented the ethanol relapse-like drinking behaviour in our cohort of female Wistar rats. The analysis of the rat body weight revealed that control animals increased their weight after the treatment but not the NAC 60 mg/kg group (Figure 43). In any case, no significant differences were detected in the body weight before and after the administration of NAC treatment, hence they did not lose weight. It was surprising that control female animals did not display the ADE phenomenon. However, results derived from experiment 1.B could be predicting this behaviour: as previously discussed as time progressed in experiment 1.B, the ethanol drinking pattern displayed by the female ADE group tended to assimilate the one displayed by the no-ADE group. Hence, in future research, (i) the application of more severe ADE/no-ADE classification criteria, (ii) the use of a larger cohort

of female Wistar rats, or (iii) shortening the experimental procedure would probably allow to overcome this limitation.

As pointed above, preclinical and clinical research has explored the potential efficacy of NAC to regulate drug-related behaviours (Brown et al., 2013; McClure et al., 2014). In the case of alcohol, few studies had previously explored the effect of NAC administration in the prevention of ethanol relapse using adequate preclinical models. Firstly, in the preclinical setting, it was reported the inefficacy of NAC to prevent the cue-induced reinstatement of ethanol seeking behaviour, under operant conditions, in male Sprague-Dawley rats. In this case, NAC was administered 2 hours before the reinstatement test at two different doses: 30 and 60 mg/kg (Weiland et al., 2015). Later, Lebourgeois and collaborators reported NAC positive effects when different ethanol-related behaviours were analysed such as: ethanol seeking or ethanol re-acquisition behaviour. Concretely, they reported that the administration of a single dose of NAC 100 mg/kg prevented ethanol-reacquisition behaviour in male Long Evans rats trained to self-administer ethanol (Lebourgeois et al., 2018). Curiously, when a different alcohol exposure protocol was used with male Wistar rats (ethanol self-administration combined with forced exposition to ethanol inhalation chambers) NAC 50 mg/kg also yielded positive effects (Lebourgeois et al., 2019). Unfortunately, in all their studies animals had limited access to ethanol (15 or 45 minutes in the operant chamber), which limits the ecological validity of this model. Besides, these procedures allow them to study the **acute effects of NAC** since it is administered only one time 1 or 2 hours before the test, but they did not provide information about the long-lasting effects that a longer

treatment of NAC could have in preventing relapse. Experimental procedures most similar to ours were those performed by Quintanilla and colleagues. They evaluated the anti-relapse efficacy of a prolonged NAC treatment under a non-operant model. Firstly, they reported that oral NAC treatment (100 mg/kg for 14 days) reduced ethanol relapse binge-like drinking, in selectively bred female rats (UChB rats) (Quintanilla et al., 2018). These results were obtained after a limited re-access period lasting for 60 min. Besides, during the realisation of the present thesis, the same group also studied the ethanol relapse-like behaviour during four days after the abstinence period. In this case, female UChB rats received NAC 40 mg/kg treatment during the last 9 days of abstinence and their results demonstrated that **the treatment was effective in preventing ethanol relapse** (Israel et al., 2021). Very recently, an additional study has analysed the anti-relapse effect of NAC under a different experimental paradigm. Concretely, male Wistar rats were exposed to intermittent access to ethanol for 4 months. Then, they were subjected to a protracted abstinence for 18 days and received a single i.p. injection of NAC 90 mg/kg 3 hours before ethanol reintroduction. According to their results, the treatment blocked the relapse-like drinking behaviour after 4 and 24 hours of ethanol reintroduction (Fredriksson et al., 2023). For the time being, no article has been found in the literature that reports NAC positive effects using both male and female rats. Unfortunately, under our experimental conditions, we could not confirm the anti-relapse effectiveness of NAC treatment in non-preferent female rats to fill this gap. Even though the differences regarding preclinical models, sex, strains and NAC dosage, all together our results are in agreement with the previous outcomes

reported by other groups contributing to the potential value of NAC as a therapeutic agent to treat AUD patients.

6.3.2 The anti-relapse effect of NAC: evaluating the underlying mechanism of action

Although there are several studies investigating the potential mechanism of action of NAC in the prevention of ethanol-motivational properties, ethanol-seeking or ethanol binge-like behaviour (Israel et al., 2021; Lebourgeois et al., 2019; Morais-Silva et al., 2016; Quintanilla et al., 2018; Schneider et al., 2015), there is a lack of studies analysing its effect during abstinence. To contribute to remedy the shortcomings in the literature, our research is focused on the NAC effects on glutamate transporters expression, oxidative stress levels and neuroinflammatory mediators that are potentially altered during protracted ethanol withdrawal. Moreover, the results of experiment 2 of the present thesis demonstrated that ethanol reintroduction is able to normalise the altered oxidative and inflammatory alterations detected after prolonged ethanol abstinence. Hence, to avoid ethanol interference, the study of the underlying mechanism of action of NAC in ethanol relapse prevention should be addressed in the absence of ethanol.

NAC antioxidant effect intercedes in the prevention of ethanol relapse

The most relevant results regarding the role of the antioxidant capability of NAC (experiment 4.1) are shown in Figure 62.

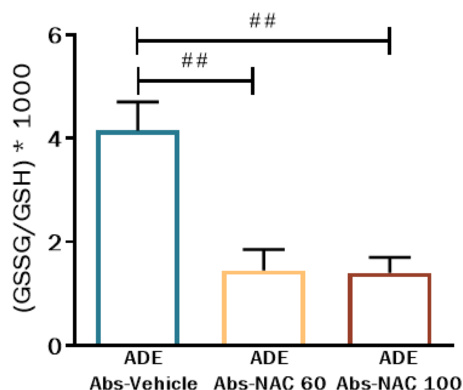


*Figure 62. The most remarkable results from experiment 4 regarding **oxidative stress status** in HIP. All experimental groups were under **abstinence conditions** when they received vehicle (blue framed bar), NAC 60 mg/kg (orange framed bar), or NAC 100 mg/kg (burgundy framed bar) treatment. Data are represented as mean ± SEM [# p<0.05; ## p<0.01]*

To date, different preclinical studies have analysed the involvement of the antioxidant effect of NAC and its role in ethanol consumption. To illustrate, the group headed by Dr. Israel demonstrated, using female UChB rats, that NAC can reduce the hippocampal oxidative stress caused by chronic ethanol consumption (Quintanilla et al., 2018). This group also reported positive antioxidant effects of NAC after 2 or 5 days of ethanol re-access after 14 days of abstinence (Quintanilla et al. 2020; Israel et al. 2021, respectively). Despite all their efforts addressed to unravel the mechanism of action of NAC, they did not analyse the effect of NAC treatment during the abstinence period i.e., during the ethanol withdrawal, without the interference of the ethanol reintroduction. In fact, the studies analysing the NAC involvement in protection against oxidative damage in the brain during abstinence are

scarce. Hence, our study has made the first attempt to address this remaining gap in the field.

After (i) detecting a tremendous increase in hippocampal oxidative stress during abstinence in vulnerable animals to ethanol relapse (experiment 3) and (ii) demonstrating the anti-relapse effect of NAC (experiment 2), the aim of experiment 4 was to explore if the anti-relapse effects of NAC could be supported by its antioxidant properties. Our data revealed that **NAC treatment at both doses (60 and 100 mg/kg) was able to fully restore the oxidative stress parameters altered after 21 days of ethanol abstinence** (Figure 48). The increased levels of GSSG and the GSSG/GSH ratio determined during abstinence were counteracted in the HIP of animals treated with NAC. Curiously, despite NAC being a source of cystine, the antioxidant NAC effect did not rely on increasing GSH levels in our experimental conditions (Figure 48 B). In contrast, a previous study demonstrated that NAC treatment increased GSH levels after cocaine exposure in rat astroglial culture (Badisa et al., 2013).

However, our data agree with the results reported by Mocellin and collaborators who demonstrated that NAC treatment was able to protect the brain of zebrafish from the oxidative damage provoked by 24 hours of ethanol withdrawal after repeated exposure (Mocelin et al., 2019). In the same line, Akhtar et al reported that ethanol-induced protein oxidation at 24h following an episode of ethanol exposure in pregnant mice was completely prevented by supplementation with NAC in both foetal and maternal brain (Akhtar et al., 2017). Remarkably, in this study NAC did not alter GSH levels. Even though our experimental conditions are distant from those used in these studies, the results

obtained point in the same direction and underline the importance of analysing alterations in oxidative status during abstinence, i.e., without the interference of ethanol and the role of NAC in preventing them.

NAC EFFECT OVER THE NEUROINFLAMMATORY CASCADE DURING ETHANOL ABSTINENCE

The most relevant results regarding the anti-inflammatory effects of NAC (experiment 4.2) are shown in Figure 63.

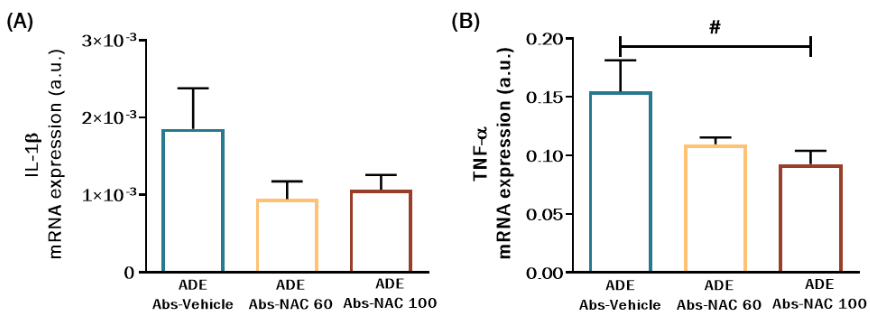


*Figure 63. The most remarkable results from experiment 4 regarding **inflammatory status** in PFC **IL-1β** (A) and **TNF-α** (B). All experimental groups were under **abstinence conditions** when they received vehicle (blue framed bar), NAC 60 mg/kg (orange framed bar), or NAC 100 mg/kg (burgundy framed bar) treatment. Data are represented as mean ± SEM [# $p<0.05$]*

The tight relationship between oxidative stress and neuroinflammation is also interesting when studying the mechanism of action of NAC. In this context, the above-mentioned studies of Dr. Israel also explored the impact of NAC administration on the astrocytic processes (a marker of neuroinflammation) in the cortex caused by chronic ethanol consumption (Quintanilla et al., 2018) or after 2 or 5 days of ethanol re-access after 14 days of abstinence (Quintanilla et al. 2020; Israel et

al. 2021, respectively). Their outcomes suggest an anti-inflammatory effect of NAC under their experimental conditions.

Since there is a dearth of studies focused on the molecular effects of NAC treatment during the abstinence period, our purpose was to check if NAC intercedes in the inflammatory process previously detected in PFC during this period (experiment 3). In order to shed light into this item, the mRNA expression of the same inflammatory markers was analysed, as previously, but after vehicle or NAC treatment. Our results showed **an increase in IL-1β and TNF-α expression during abstinence that is prevented by NAC in the case of TNF-α** and nearly prevented in the case of IL-1β (Figure 63). In relation to NfκB (Figure 50 B), an appreciable increase during abstinence can also be observed, and it seems to be alleviated by NAC, although statistical analysis did not detect significant differences. Our outcomes correlate with the previous ones reported by Schneider and collaborators who tested the effect of NAC on the neuroinflammation caused by 24 hours of ethanol abstinence. They found that NAC 60 and 90 mg/kg restored the levels of inflammatory markers in HIP and frontal cortex of male Wistar rats (Schneider et al., 2017).

Regarding the pathway through which NAC exerts its anti-inflammatory effect, a study suggested that it could be directly mediated by the suppression of IKKα and IKKβ kinases activity which play a role in the initiation of the immune cascade (Oka et al., 2000). Nonetheless, other mechanisms may be plausible. For instance, NAC can prevent the increase in ROS levels which would also avoid the subsequent NfKβ activity that is associated with the expression of IL-1β and TNF-α. To

test this possibility, further research is needed to measure ROS levels in the PFC during the abstinence period.

NAC DOES NOT EXERT AN EFFECT OVER THE GLUTAMATERGIC PROTEIN EXPRESSION IN THE STRIATUM

NAC capability to upregulate GLT1 and xCT levels altered by cocaine consumption has been repeatedly demonstrated and proposed as a potential mechanism of action of this drug in the field of addiction research (Ducret et al., 2016; Knackstedt et al., 2010; Reissner et al., 2015). Nonetheless, the ability of NAC to alter protein expression in nucleus accumbens after chronic ethanol intake has been less studied. Since a diminished GLT1 and GLAST expression was detected by our group in the nucleus accumbens during abstinence, we judged that the upregulation of their expression could be another plausible mechanism that underlie the anti-relapse effect of NAC. However, the present results have shown **that NAC did not affect GLT1, GLAST nor xCT protein expression under our experimental conditions**, based on a long-term drinking history with several deprivation periods (Figure 52 and Figure 53). Hitherto, preclinical studies that explore the effects of NAC and ethanol consumption on the expression of these proteins in nucleus accumbens are scarce. Curiously, Lebourgeois and collaborators found that NAC induced a 28% decrease in the expression of GLT1 in male Wistar rats that self-administered ethanol although no alteration in xCT expression was observed (Lebourgeois et al., 2019); the lack of effect on xCT expression was corroborated in a study performed in male mice exposed to ethanol and treated with NAC (Morais-Silva et al., 2016). Other authors have analysed the effect of NAC on GLT1 expression in PFC in female UChB rats chronically exposed to ethanol consumption.

Their results are in close agreement with ours, given that they reported that, on the one hand, chronic ethanol exposure significantly reduced by 50% the expression of GLT1 in PFC and, on the other hand, NAC treatment, administered during 9 days of the alcohol abstinence phase, did not modify this reduction (Israel et al., 2021).

According to our results, NAC treatment during abstinence was not able to reverse glutamatergic alterations observed in nucleus accumbens. Consequently, **our reported anti-relapse effect of NAC (experiment 2) does not seem to be linked with its effect on these glutamatergic proteins' expression.** Nonetheless, it is important to highlight that the literature indicates that GLT1, GLAST activities are altered by oxidant environment (Trotti et al., 1998) and that system xC- activity could be potentially reduced under prooxidant conditions (Ghasemitarei et al., 2019). Therefore, in future research it should be explored whether the anti-relapse effect of NAC, that could be related with its effects on the striatal glutamate signalling, may be mediated through the alteration of the activity of these proteins rather than the available amount of the transporter.

NAC TREATMENT DURING ABSTINENCE SEEMS TO MIMIC THE EFFECTS DISPLAYED BY ETHANOL REINTRODUCTION ON OXIDATIVE AND NEUROINFLAMMATORY STATUS.

An interesting aspect reported in the present thesis is the role of ethanol reintroduction at several neurobiological levels such as oxidative and neuroinflammation status. In particular, we have evidenced that ethanol is able to correct the prooxidant and inflammatory status triggered by 21 days of ethanol abstinence. Curiously, according to the results obtained in experiment 4, NAC

seems to mimic some of the effects observed after ethanol reintroduction. An in-depth analysis of both effects, i.e., ethanol reintroduction and NAC administration during abstinence, show a similar profile (Table 15).

		Percentage of reduction in comparison with vehicle (abstinence) group		
	Parameter	NAC 60 mg/kg	NAC 100 mg/kg	Ethanol re-introduction
Oxidative stress	GSSG	61%*	62%*	89%*
	GSSG/GSH	65%*	66%*	84%*
Inflammatory markers	IL-1β	53%	42%	79%*
	TNF-α	30%	40%*	56%*

Table 15. Comparative effects of NAC (60 or 100 mg/kg) and ethanol reintroduction on the reduction of oxidative and neuroinflammatory markers. Results are expressed as percentage of reduction in comparison with the vehicle (abstinence) group. (*) denotes statistical differences with respect to the abstinence group.

As can be seen in Table 15, in the case of oxidative status, NAC (both doses) provoked a significant reduction of around 65 % in GSSG/GSH levels in relation to the abstinence (vehicle) group, whereas ethanol reintroduction induced an 84 % decrease. Regarding inflammatory mediators, the results were qualitatively similar. In all cases, ethanol effects are always more pronounced than that observed after NAC administration.

Consequently, it could be hypothesised that during abstinence NAC administration could exert the same role as ethanol reintroduction, thus reducing the propensity for ethanol consumption relapse. Additionally, as discussed in the following chapter, we have also demonstrated that NAC, acutely administered, is able to mimic some of the neurobehavioural effects provoked by the local administration of ethanol in the MCLS. These striking data suggest that further experiments exploring additional mechanistic approaches should be performed to fully decipher the role of NAC to prevent neurobiological alterations induced during relapse.

6.3.3 Future research

The present study provides evidence **that (i) chronic NAC treatment during long-term abstinence can prevent ethanol relapse-like drinking behaviour and (ii) the neurobiological alterations related to brain oxidative stress and neuroinflammation observed after three weeks of ethanol abstinence in long-term ethanol-experienced male rats, can be alleviated by chronic NAC administration during this period.** Conversely, NAC does not seem to blunt the modifications observed in some astrocytic glutamate transporters (downregulation of GLT1 and GLAST in nucleus accumbens) during abstinence under the same experimental conditions. These findings are crucial to deepen in the understanding of the NAC mechanism of action.

Despite these preclinical results, **better clinical outcomes** could be obtained if NAC treatment were combined with other pharmacological agents, addressed not only to maintain abstinence conditions, but also to promote the drug intake cessation (McClure et al., 2014). The

combination of treatments with different mechanisms of action can improve the effectiveness of therapies (Quintanilla et al., 2020). In this line, the group headed by Dr. Israel has already studied, in a preclinical model with UChB female rats, the benefits of the treatment with NAC and acetylsalicylic acid (ASA) (Quintanilla et al., 2020) or a combined treatment of NAC, ASA and a bilateral vagotomy (Quintanilla et al., 2022). They reported positive effects in ethanol intake and relapse-like drinking behaviour. According to our outcomes, the combination of NAC treatment with a drug that increases GLT1 or GLAST protein expression, such as ceftriaxone (Rao, et al., 2015b), could provide better clinical results.

Even though valuable results have emerged from our research, it is mandatory to complement this data with **preclinical studies conducted with a female cohort of rats.** The study of SUDs in women has been largely ignored and recent research has been drawing interesting conclusions, such as the association between menopausal symptoms and lower rates of smoking abstinence (Copeland et al., 2017). Moreover, steroidal hormones play a role in different pathways related with the studies presented herein, such as immune modulation (Monasterio et al., 2014) or glutamate homeostasis e.g., E2 increases GLT1 expression in astroglia (Lee et al., 2012). Fortunately, the upsurge in AUD studies with gender perspective is evident. In fact, the described sex differences in behavioural risk factors and neural systems implicated in AUD are collected in a recent review (Flores-Bonilla & Richardson, 2020). In conclusion, it should not be overlooked the impact of sex differences and reproductive life cycle with SUDs, especially when developing new pharmacotherapies.

ACUTE EFFECTS OF NAC IN THE MCLS

Section adapted from **The Effects of N-Acetylcysteine on the Rat Mesocorticolimbic Pathway: Role of mGluR5 Receptors and Interaction with Ethanol** Fernández-Rodríguez, S., Esposito-Zapero, C., Zornoza, T., Polache, A., Granero, L., & Cano-Cebrián, M. J. Published in *Pharmaceuticals* 14.6 (2021): 593.

This section pursued the objective of giving a preliminary insight into the potential effects of NAC on the MCLS. Concretely, several studies, using immunohistochemical or behavioural procedures, were carried out to make an initial approach to the exploration of the acute effects of NAC in nucleus accumbens neurotransmission.

6.4 ACUTE EFFECT OF N-ACETYLCYSTEINE ON THE MESOCORTICOLIMBIC PATHWAY

Given that NAC is a prodrug of cysteine and cystine (its dimeric form), its presence could alter the activation of MCLS through two plausible ways: (i) its administration has been associated with the increase of extracellular glutamate levels through its action over the system xC- antiporter and (ii) it is feasible that NAC, or its derivatives, could react non-enzymatically with ACD to form stable, non- toxic adducts altering ethanol's rewarding properties. In fact, as above-mentioned, Kupchick and collaborators, performed *in vitro* studies and found that the augmentation of extracellular glutamate induced by the presence of low doses of NAC may produce a preferent activation of presynaptic

mGluR2/3 inhibiting glutamate transmission onto nucleus accumbens. Conversely, a higher increase in glutamate levels, induced by the presence of higher doses of NAC, should counteract this effect by stimulating also the relatively lower affinity mGluR5 (Kupchik et al. 2012). This dose-dependent effect of NAC on glutamatergic transmission in the nucleus accumbens points out the relevance of selecting the appropriate dose of NAC to obtain concrete clinical outcomes. As we have discussed above, this study supports our results from experiment 2.B which reflected an inverted U-shaped dose-effect curve of NAC on preventing ethanol relapse-like drinking behaviour.

Thus, we initiated a set of *in vivo* experiments to explore the effects of different NAC doses, after acute administration, on the activation of the MCLS and the underlying mechanisms. The effect of NAC on ethanol's rewarding properties in the MCLS, probably mediated through the inactivation of the ACD, was also explored. Hence, the aims of the present experiments were:

-Explore the effect of high and low systemic doses of NAC on the activation of the MCLS, probably triggered by synaptic glutamatergic transmission onto the nucleus accumbens neurons.

-Analyse the influence of MTEP, a selective negative allosteric modulator of mGluR5.

- Explore the effects of the administration of a high NAC dose (120 mg/kg) on the rewarding properties of ethanol. The neurochemical and behavioural activation of the mesocorticolimbic pathway was induced by the intra-VTA administration of 150 nmol of ethanol.

6.4.1. MTEP SUPPRESS THE ACTIVATION OF MESOCORTICOLIMBIC SYSTEM INDUCED BY A HIGH DOSE OF N-ACETYLCYSTEINE.

As described in the literature, the pharmacological actions of NAC may include the restoration of the glutamatergic function disrupted in a myriad of disorders such as several SUDs (McClure et al., 2014). However, the results obtained in the present thesis (experiment 4.3) reveals that, under our experimental conditions, NAC chronically administered did not normalise the expression of GLT1 and GLAST, that was downregulated in nucleus accumbens during protracted abstinence. On the other hand, as NAC is known to induce an increase in endogenous glutamate levels (Dean et al., 2011), deepening in the study of the mechanisms involved in the effects of NAC into the glutamatergic signalling in the MCLS may help to better understand its complex mechanism of action. In this sense, some authors defend that NAC may interact with glutamate transporters, such as the astrocytic antiporter system xC-, which is able to increase glutamate levels in the extrasynaptic space in the nucleus accumbens (Massie et al., 2015). In the present thesis, we tried to analyse NAC modulatory effects on synaptic glutamate transmission. Firstly, we explored the activating effects of high and low systemic doses of NAC on the synaptic transmission onto the nucleus accumbens neurons. Secondly, we explored the influence of MTEP. For both purposes, we used **cFOS early gene expression** as a tool to measure the activation of the MCLS. This technique has been extensively validated in our laboratory (Campos-Jurado et al., 2019; Zornoza et al., 2005). The measurement of cFOS-IR cell increased expression allows to determine the activation of the VTA in its main projection areas, such as the nucleus accumbens, as

well as the metabolic consequences of the induced postsynaptic activity (Jaworski et al., 2018). Thus, several neurochemical studies have shown that the electrical or chemical stimulation of glutamatergic areas with afferences in the nucleus accumbens is accompanied by a significant and persistent induction of cFOS early gene expression in this area (Bardgett & Henry, 1999).

According to our results, i.p. injection of 30 mg/kg of NAC (low dose) slightly increases the expression of cFOS-IR cells in nucleus accumbens, while a **higher dose (120 mg/kg) clearly increases their expression compared to the control group** (Veh/Sal). The simultaneous administration of MTEP (0.1 mg/kg) apparently blocks this increase in cFOS-IR cell expression; however, there is no relevant effect when it is co-administered with the lower dose (Figure 55). It could be hypothesised that the i.p. administration of a high dose of NAC provokes an important increase—especially in extrasynaptic glutamate levels—that would lead to an activation of the nucleus accumbens medium spiny neurons (MSNs) and would consequently increase cFOS-IR cell expression in this area. Our results when using MTEP are in agreement with those reported by Kupchik et al. and seem to confirm the dual role displayed by extracellular glutamate in nucleus accumbens activation. In their electrophysiological studies on rat brain slices, the authors showed that 5 mol/L of MTEP did not alter the decreased excitatory postsynaptic currents (EPSCs) amplitude evoked by 0.5 mol/L of NAC (low dose), whereas the same concentration of MTEP was able to counteract the increase of 500 mol/L of NAC-(high dose) induced EPSCs amplitude (Kupchik et al., 2012). These authors concluded that the excitatory effects of a high NAC dose were mediated

by post-synaptic mGluR5. In the present thesis, we have confirmed these results but under *in vivo* conditions. Concretely, 0.1 mg/kg of MTEP was able to block the increase in cFOS-IR cell expression evoked by a high dose of NAC. Moreover, **the low NAC dose had no effect on cFOS-IR cell expression in the nucleus accumbens.** A comparable result was obtained by Laverde and colleagues when they were studying the effect of NAC on ethanol rewarding's properties. For this aim they used the CPP paradigm. In the preclinical setting, CPP is one of the paradigms widely accepted to approach context-drug associations. Thus, rats can develop a preference for an environment due to the association of this context with the reinforcing properties elicited by the administered drug through the activation of the MCLS. Curiously, they detected that NAC *per se* induced CPP when a 120 mg/kg dose was assayed but not 60 mg/kg, suggesting that the high dose assayed induces the activation of the MCLS (Laverde et al., 2021).

To sum up, our results seem to indicate that the effect of a high dose of NAC could be mediated not only through the activation of the presynaptic mGluR2/3, but also through the postsynaptic mGluR5. Hence, it could be plausible that the blockade of the postsynaptic receptors (mGluR5) may lead to the abolition of this activating effect. All in all, further experiments involving (i) lower doses than NAC 30 mg/kg so as to avoid an interaction with mGluR5 and (ii) other antagonists of mGluRs type I and II are necessary to corroborate this hypothesis.

6.4.2 NAC BLUNTED THE ACTIVATION OF THE MESOCORTICOLIMBIC SYSTEM INDUCED BY LOCAL ETHANOL ADMINISTRATION

In this set of experiments, we aimed to explore the potential effects of NAC in the rewarding properties of ethanol. For this purpose, 150 nmol of ethanol were directly administered in the posterior VTA (pVTA) of male Wistar rats. As previously reported, this treatment induces the excitation of VTA dopaminergic neurons and has been widely correlated with increases in animal exploring activity (Sánchez-Catalán et al., 2009). This procedure has been extensively used in our laboratory in order to study the basis of the reinforcing properties of ethanol (Martí-Prats et al., 2010, 2013, 2015b). Two different experimental procedures were used to measure the activation of the MCLS: (i) the evaluation of the locomotor activity displayed by the rats, that can be considered an indirect procedure to determine MCLS activation, and (ii) the quantification of cFOS expression in nucleus accumbens that can be considered a direct determination of the MCLS activation.

As shown in Figure 59, **not only the treatment with ethanol, but also the treatment with NAC 120 mg/kg significantly increased cFOS expression in nucleus accumbens. Surprisingly, the co-administration of NAC and ethanol did not have any effect on cFOS expression,** suggesting a plausible interaction between both molecules. As previously commented, these effects were also indirectly studied by measuring the locomotor activity displayed by the rat of each experimental group. Although no significant differences were determined, the obtained results showed the same trend as the cFOS quantification. The results of the locomotor activity experiment (Figure 57) suggest that **the direct**

infusion of ethanol into the pVTA results in the induction of the locomotor activity displayed by the rats, although this effect was not statistically significant in the present set of experiments. It should be remembered that the significant increase of the locomotor activity after the intra-VTA administration of 150 nmol of ethanol has been repeatedly reported by our group (Martí-Prats et al., 2010; Sánchez-Catalán et al., 2009). Moreover, NAC 120 mg/kg seems to increase the locomotor activity by itself. However, as previously discussed, **the co-administration of NAC and ethanol did not alter the locomotor activity displayed by our rats.** From a critical point of view, we suggest that the lack of statistical differences in the present experiment is probably due to the great variability in the behavioural responses displayed by the animals. An increased number of animals included in the present study would allow us to overcome this limitation.

Again, our results are in close agreement with those reported by Laverde and colleagues: on the one hand, they reported that either ethanol (0.1 g/kg) or NAC 120 mg/kg *per se* induced CPP i.e., when either ethanol or NAC were administered during the conditioning phase they induced a drug-reward related behaviour. On the other hand, NAC 120 mg/kg was able to block the manifestation of ethanol CPP when both substances were daily co-administered during the conditioning phase, suggesting that both substances could interact with each other leading to a neutralisation of their reinforcing properties. Finally, when the effect of a single dose of NAC 120 mg/kg on the ethanol-induced CPP was evaluated during the postconditioning phase (without ethanol presence) the CPP was not altered (Laverde et al., 2021). The authors discussed that the development of ethanol CPP is blocked by daily NAC

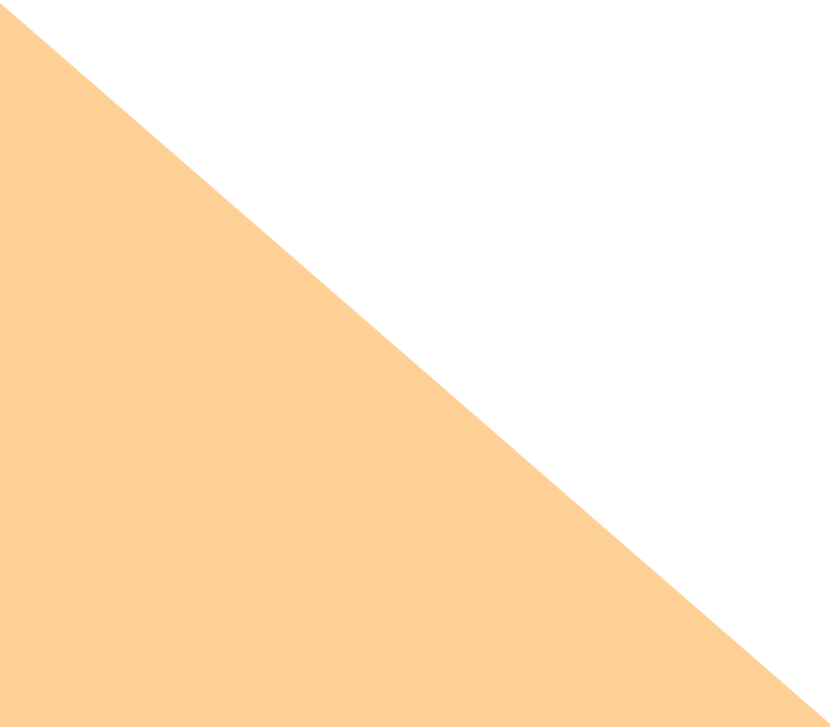
administration during the conditioning phase, while acute NAC administration the day of the test (in absence of ethanol) does not affect the expression of ethanol CPP. In the same line, Morais-Silva and collaborators reported that NAC 120 mg/kg, but not 60 mg/kg, treatment was able to block the behavioural sensitisation induced by ethanol administration in Swiss mice (Morais-Silva et al., 2016). Taken together, it could be hypothesised that both drugs may interact when simultaneously administered, so that the administration of one drug is able to counteract the effect produced by the other. In any case, to fully understand this potential interaction further experiments are needed. However, it could be interesting to give a possible explanation about what may happen when NAC and ethanol are co-administered. Concretely, a feasible mechanism that could underlie this scenario is **the potential sequestration of ACD through NAC.** It is widely known that the brain metabolism of ethanol produces ACD (Hipolito et al. 2007; Correa et al. 2012), a neuropharmacological active substance which has reinforcing properties by itself (Correa et al., 2012; Peana et al., 2016). There is a large body of literature that evidences that this molecule is responsible, at least in part, of the neurobehavioural effects associated with ethanol consumption. These effects are mediated through the activation of the MCLS (Foddai et al., 2004; Melis et al., 2007). In fact, the literature evinces that ACD was orally self-administered under operant procedures and its oral self-administration could be prevented by **L-cysteine** (Peana et al., 2012), a sulfhydryl amino acid agent that binds covalently with ACD producing a stable compound (Salaspuro et al., 2002). Besides, L-cysteine, through the sequestration of ACD is able to block the acquisition, extinction and reinstatement of ethanol consumption under operant conditions

(Peana et al., 2009, 2010, 2013). Given that NAC is a cystine and cysteine prodrug it is plausible that its administration also provokes the sequestration of ACD. Regarding experiment 5.2, the experimental design allowed that ethanol locally administered in VTA could be transformed into ACD, since the required enzymes for its metabolism are present in VTA: catalase and CYP2E1 (Sánchez-Catalán et al., 2008; Zimatkin et al., 2006). Like previous data obtained in our laboratory (Martí-Prats et al., 2010; Sánchez-Catalán et al., 2009), the present results revealed that the microinjection of 150 nmol of ethanol in the VTA causes an augmentation in the activation of the MCLS that could be due to the presence of ACD and/or its metabolite derivative salsolinol (Hipólito et al., 2012). As the co-administration with NAC 120 mg/kg abolishes this increase, it is plausible **that NAC may interact with the ACD locally generated in VTA to prevent the activation of the MCLS**. The chemical inactivation of ACD also underlies the anti-relapse effect of other treatments studied in our own laboratory such as D-Penicillamine (Martí-Prats et al., 2015a; Orrico et al., 2013). In this sense, we wonder if the potential inactivation of ACD through the administration of NAC, may be considered an additional mechanism that also participates in the capacity of NAC to prevent the alcohol relapse demonstrated in experiment 2.

Finally, in our opinion our results can also be interpreted by an alternative point of view. Several studies have postulated that the nucleus accumbens responses evoked by glutamate are attenuated when DA is present. Cepeda and colleagues reported that the responses evoked by the iontophoretic application of glutamate in rat striatum slices were significantly attenuated when DA was applied

(Cepeda et al., 1993). More recent studies have postulated that long-term depression (LTD) of the MSNs is produced when high concentrations of DA are released in the nucleus accumbens, and that this effect is mediated by D1 receptors (Nicola et al., 2000; Surmeier et al., 2007; Wang et al., 2006). In addition, there is general agreement that this form of LTD is induced postsynaptically and depends upon the activation of mGluR type I (Brebner et al., 2005; Centonze et al., 2001; Lovinger et al., 2003). Thus, one plausible explanation of the observed results under our experimental conditions could involve the interaction between glutamate and DA release in the nucleus accumbens. It is well known that the intra-VTA administration of ethanol can induce a phasic DA release in the nucleus accumbens (Ding et al., 2009), and, as stated above, the systemic administration of a high dose of NAC can also produce an increase in extrasynaptic glutamate. Therefore, the release of glutamate in presence of increased levels of DA could lead to the LTD of the MSNs in the nucleus accumbens and the suppression of cFOS expression in this brain area. It is obvious that further experiments are needed to corroborate this alternative hypothesis.

7. CONCLUSIONS



The present thesis evinces the importance of the brain oxidative disbalance and the neuroinflammatory status in the ethanol relapse-like drinking behaviour in animals chronically exposed to ethanol consumption under the ADE paradigm. Furthermore, we propose a therapeutic strategy, NAC administration, that is able to prevent the ethanol relapse-like drinking behaviour through its effects on the above-mentioned alterations.

The most remarkable conclusions drawn from the data obtained in this thesis are:

C1. The preclinical ADE model allows to identify two subpopulations of male and female Wistar rats that differ in their vulnerability to ethanol relapse and display different patterns of ethanol intake and ethanol preference.

C2. NAC effectively prevents the ethanol relapse-like drinking behaviour displayed by vulnerable Wistar rats. Under our experimental conditions, the chronic administration of NAC 60 mg/kg once per day provides the best preclinical outcomes.

C3. After experiencing a prolonged abstinence period (21 days), the oxidative status in the HIP and the neuroinflammatory status in the PFC of male rats remain altered, but only in animals that repeatedly displayed ethanol relapse-like drinking behaviour, possibly being a plausible key in the induction of the craving that will lead to the relapse process.

C4. The levels of GLT1 and GLAST in the nucleus accumbens of vulnerable animals are diminished during abstinence.

C5. The re-access to ethanol consumption (24 hours) alleviates the oxidative stress and the neuroinflammation detected during prolonged abstinence in vulnerable animals.

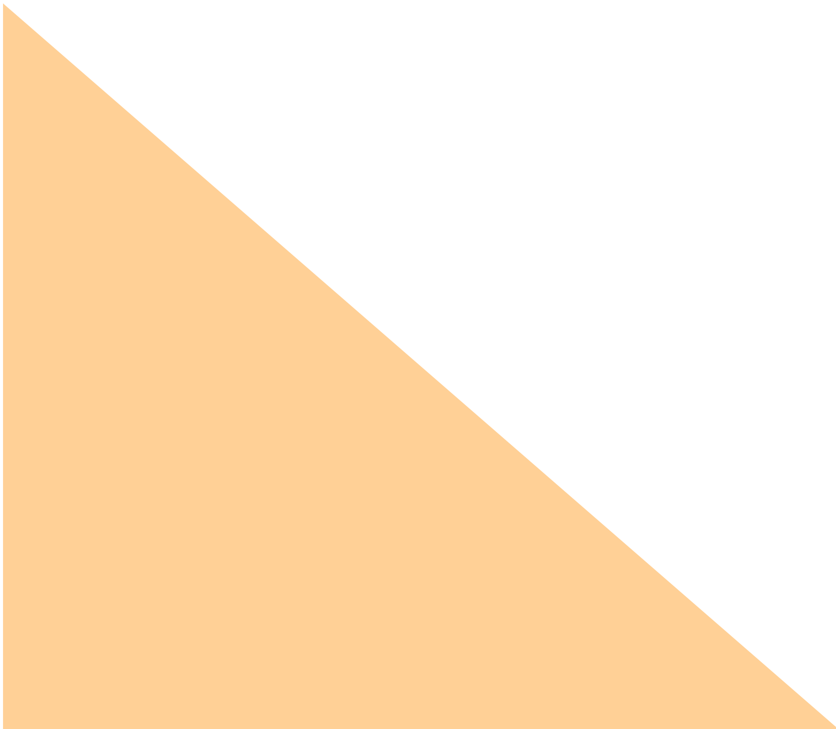
C6. NAC treatment during protracted abstinence restores the oxidative stress levels of HIP and the levels of TNF-α in PFC, suggesting that its anti-relapse effect is due, at least in part, to its antioxidant and anti-inflammatory capabilities.

C7. NAC does not affect the protein expression of GLT1, GLAST and xCT in the striatum of vulnerable animals when it is administered during protracted abstinence.

C8. Acute NAC treatment has a dose-dependent effect on glutamatergic transmission. Only a high dose activates the glutamatergic transmission leading to a significant increase in cFOS-IR cell expression in the nucleus accumbens. The negative allosteric modulator of mGluR5 (MTEP) is able to abolish this effect, suggesting that the activating effect of NAC could be mediated by mGluR5.

C9. The ethanol-induced increase in the expression of cFOS-IR cells in nucleus accumbens is attenuated by the co-administration of a high dose of NAC.

8. RESUM



Introducció

El **consum d'alcohol** està àmpliament estès en moltes cultures, malgrat ser una substància psicoactiva el consum de la qual pot desencadenar en un trastorn per consum d'alcohol (TCA) (World Health Organization, 2018). Segons l'Organització Mundial de la Salut, un 5.1% de la població pateix TCA, una patologia crònica i recidivant en la qual el pacient persisteix en el consum de la droga malgrat ser conscient de les conseqüències negatives que comporta. Des d'un punt de vista clínic, un dels principals problemes per a un tractament adequat dels trastorns per consum de drogues és, l'elevada taxa de **recaigudes**, les quals poden desencadenar-se fins i tot després de diversos anys sense consumir la droga (Kalivas, 2009). L'**abstinència** és per tant una etapa clau en la qual cada pacient pot ser més o menys vulnerable a la recaiguda. Hui dia, les neuroadaptacions que potencialment poden ocórrer en aquest període, i que precedeixen a la recaiguda en el consum d'alcohol, no es coneixen en profunditat. Aprofundir en el coneixement d'aquests canvis és clau per a comprendre la neurobiologia del procés de la recaiguda i poder dissenyar tractaments més eficaços per a la seua prevenció (Cannella et al., 2019).

A conseqüència de la presència de reforçadors naturals com són el menjar, aigua o sexe, el sistema mesocorticolímbic (SMCL), s'activa i es produeix un alliberament de dopamina en el nucli accumbens. El consum de drogues causa una activació aberrant del sistema dopaminèrgic que pot desembocar en els passos inicials del desenvolupament d'un trastorn addictiu (Kalivas, 2009). D'altra banda, el consum de substàncies d'abús causa altres neuroadaptacions en

diferents àrees del SMCL, que es manifesten fins i tot després del cessament del consum. De fet, el període d'**abstinència** a diferents drogues, entre elles l'alcohol, està relacionat amb un augment aberrant de la **senyalització glutamatèrgica** en nucli accumbens (Alasmari et al., 2018).

Mantenir les condicions basals de glutamat extracel·lular és important atès que la sobreactivació dels receptors cel·lulars de glutamat desencadena un estat patològic conegut com excitotoxicitat (Trotti et al., 1998). El *glutamate transporter 1* (**GLT1**) i *el glutamate aspartate transporter* (**GLAST**) són recaptadors de glutamat sinàptic. Estan localitzats en astròcits i són els principals responsables de mantenir les concentracions de glutamat sinàptic en àrees com el còrtex, hipocamp (HIP), estriat (STM) o cerebel (Lehre et al. 1995; Lehre & Danbolt 1998). Una altra proteïna amb un important impacte en els nivells extracel·lulars de glutamat és el transportador d'aminoàcids **sistema xC-.** Es tracta d'un bescanviador de cistina-glutamat que s'expressa principalment en astròcits i microglia i que intercanvia cistina extracel·lular per glutamat intracel·lular, la qual cosa fa d'aquest sistema el principal proveïdor de glutamat extracel·lular en zones com a nucli accumbens, STM o HIP (Massie et al., 2015). La seua activitat té dues conseqüències directes: d'una banda, proveeix de cisteïna a l'astròcit per a la síntesi de glutatió (GSH) i d'altra banda augmenta els nivells extra sinàptics de glutamat (Berríos-Cárcamo et al. 2020), activant així els receptors metabotròpics de glutamat (mGluR). Així pues, l'activaació del mGluR2/3 presinàptic, inhibeix l'alliberament de glutamat sinàptic, i la del mGluR5 estimula l'activació de la neurona postsinàptica (Kupchick et al., 2012). La transmissió glutamatèrgica

exacerbada està relacionada amb la tolerància i dependència a l'etanol (Littleton, 1995) i també es relaciona amb el procés de recaiguda segons la **hipòtesi glutamatèrgica de l'addicció a drogues.** Segons aquesta, les projeccions glutamatèrgiques de PFC, amígdala (AMG) i HIP a nucli accumbens s'activen davant pistes contextuals associades al consum de drogues, causant alliberament de glutamat en nucli accumbens i la conseqüent recaiguda en el consum de la droga (Kalivas et al., 2009). Nombrosos estudis han demostrat que en condicions d'exposició contínua i intermitent al consum d'**etanol** i l'abstinència primerenca, hi ha un augment dels nivells de glutamat en nucli accumbens (Das et al., 2005; 2015; Pati et al., 2016; Saellstroem Baum et al., 2006). Nombrosos estudis també han demostrat que el consum crònic d'etanol redueix els nivells d'expressió proteica de GLT1 en aquesta àrea mentre que els nivells de xCT i GLAST no es veuen afectats o es redueixen (Alhaddad et al., 2014; Das et al., 2015; Ding et al., 2013; Hakami et al., 2016; Sari et al., 2013). No obstant això, no hi ha evidències clares de com el període d'abstinència prolongada afecta a l'expressió d'aquestes proteïnes en nucli accumbens.

Una elevada transmissió glutamatèrgica sobreactiva la neurona postsinàptica causant un procés de excitotoxicitat amb una elevada producció d'espècies reactives d'oxigen (ROS per les seues sigles en anglés). Aquest augment es veu pal·liat per la presència del GSH, que en condicions prooxidants s'oxida a GSSG (Aoyama, 2021). En general, el consum de drogues suposa un augment de **l'estrès oxidatiu** a nivell cerebral a causa del metabolisme de la dopamina alliberada i el metabolisme de la pròpia droga (Womersley et al., 2019). En el cas concret de l'etanol, el seu consum s'ha vinculat amb augment de

diferents marcadors d'estrès oxidatiu en el còrtex i HIP principalment (Quintanilla et al., 2018; Reddy et al., 2013). A més, durant el període d'abstinència primerenca a l'etanol s'han detectat desequilibris en els nivells d'estrès oxidatiu en HIP (Elibol-Ca et al., 2011; Jung et al., 2008). No obstant això, els estudis centrats en un període d'abstinència tardana són pràcticament inexistents. Per això, encara és necessari explorar els nivells d'estrès oxidatiu durant l'abstinència prolongada i comprovar si les possibles condicions pro-oxidants poden estar relacionades amb el posterior procés de recaiguda.

Per altra banda, l'augment de l'estrès oxidatiu va lligat irremeiablement a **processos neuroinflamatorios** ja que la presència de ROS activa el factor de transcripció kappa B (NfκB) que estimula l'alliberament de molècules pro-inflamatòries des de les cèl·lules microglials. Aquest procés pot desencadenar-se també per la interacció directa de les drogues d'abús, com l'etanol, a través dels *receptors toll-like* (TLRs) (Berríos-Cárcamo et al. 2020). De fet, diferents estudis clínics i preclínics han demostrat que l'exposició a etanol augmenta els mediadors neuroinflamatoris, sent el còrtex cerebral una de les àrees més afectades (He & Crews, 2009; Sanchez-Alavez et al. 2019). En relació al període d'abstinència, en la literatura es troben evidències preclíniques sobre processos inflamatoris cerebrals (Knapp et al. 2016; Vetreno et al., 2013). Així i tot, els estudis són escassos i poc concloents, per la qual cosa la comunitat científica ha reclamat recentment la necessitat d'explorar com els processos neuroinflamatoris varien en les diferents etapes del cicle de l'addicció i si els possibles canvis durant un període d'abstinència prolongada

poden estar relacionats amb el comportament de cerca de la droga previ a la recaiguda (Gipson et al., 2021).

Un dels models animals més adequats per a l'estudi de la recaiguda en el consum d'etanol i, per tant, per a l'estudi de les alteracions subjacents en aquest comportament, és el model basat en l'efecte de privació a l'alcohol, també conegut com a ***alcohol deprivation effect* (ADE)** en terminologia anglosaxona. El model ADE és un model animal d'exposició continuada a l'alcohol amb fases repetides d'abstinència en el qual es quantifica el denominat fenomen ADE. Aquest fenomen consisteix en l'increment temporal en el consum d'alcohol que es produeix en l'animal d'experimentació després d'un període d'abstinència forçada (Sinclair & Senter, 1968). S'ha suggerit que el ADE està relacionat amb l'alcohol craving o desig compulsiu pels efectes de l'etanol prèviament experimentats i que constituiria un determinant fonamental per a la recaiguda després dels períodes d'abstinència. Per això, **la manifestació del fenomen ADE es considera un comportament similar a la recaiguda en humans.** Aquest model animal comprèn les diferents fases del cicle de l'addicció incloent el procés d'adquisició i manteniment del consum de la droga, l'abstinència i la recaiguda, la qual cosa justifica la seua validesa aparent (Bell et al., 2017; Leong et al., 2018). A més, els fàrmacs comercialitzats actualment com a tractaments anti-recaiguda per al consum d'etanol com la naltrexona o l'acamprosato també redueixen la manifestació del fenomen ADE en aquest model, la qual cosa evidencia la seua validesa predictiva (Bachteler et al. 2005; Hölter & Spanagel, 1999). Per tot això, aquest model és una eina idònia i àmpliament utilitzada per a l'estudi de la recaiguda en el consum

d'etanol, així com per a l'avaluació de l'eficàcia de nous fàrmacs anti-recaiguda (Spanagel, 2017).

La farmacoteràpia actual dirigida a la prevenció de les recaigudes en el consum d'alcohol ha demostrat ser poc eficient i amb una modesta taxa d'adherència a causa dels seus efectes adversos (Ch'Ng & Lawrence, 2018). Per això, la comunitat científica continua dirigint nombrosos esforços al desenvolupament de nous fàrmacs en aquest àmbit. Un dels candidats més estudiats és la N-acetilcisteïna (NAC), un profàrmac de la cisteïna amb propietats glutamatèrgiques, antioxidants i antiinflamatòries (McClure et al., 2014). Els assajos preclínics han mostrat que l'administració sistèmica de NAC és capaç de prevenir el restabliment del comportament de cerca de la cocaïna, l'heroïna i la nicotina (Amen et al., 2011; Moro et al., 2018; Zhou & Kalivas, 2008). Aquests efectes s'han atribuït, almenys en part, a la capacitat del tractament crònic amb NAC per a restaurar la neurotransmissió glutamatèrgica en el nucli accumbens (Kalivas, 2009), encara que també podrien estar associats a la seua capacitat antioxidant i antiinflamatòria (Smaga et al., 2021). A pesar que els estudis preclínics que avaluen els efectes de la NAC en el consum d'etanol i recaiguda són nombrosos, trobem algunes limitacions en la literatura. D'una banda, els estudis publicats utilitzen principalment rates seleccionades pel seu elevat consum d'alcohol (Quintanilla et al., 2016, 2018), la qual cosa podria disminuir el poder traslacional dels resultats obtinguts. D'altra banda, en la majoria dels articles publicats s'analitza l'efecte d'una dosi única de NAC o s'utilitzen models experimentals que no han mostrat robustesa suficient en la investigació preclínica (Lebourgeois et al., 2018, 2019). Finalment, els

estudis que avaluen les alteracions produïdes per l'etanol durant el període d'abstinència tardana, i analitzen l'eficàcia de la NAC en la possible restauració d'aquestes alteracions, són molt escassos o fins i tot inexistents. Així doncs, la present tesi s'ha dissenyat amb l'objectiu d'aprofundir en la neurobiologia del procés de la recaiguda, així com en l'avaluació de l'eficàcia de la NAC com a tractament per a prevenir-la. Per a això, s'han utilitzat rates Wistar no seleccionades pel seu alt consum d'etanol i un model preclínic robust com és el model ADE. A més, atès que els estudis en el camp de l'addicció realitzats amb femelles són escassos, malgrat la importància d'estudis amb perspectiva de gènere (Gipson et al. 2021), i que estudis previs han trobat diferències en els efectes psicofarmacològics de la NAC en funció del sexe (Goenaga et al., 2020; Monte et al., 2020), els experiments comportamentals d'aquest treball inclouen una cohort d'animals mascle i una cohort d'animals femella.

Objectius

Els experiments que s'inclouen en la present tesi s'han dissenyat per a tractar de donar resposta als següents objectius:

- Caracteritzar del comportament de recaiguda en una població no preferent de rates Wistar exposades de forma prolongada al consum voluntari d'etanol sota un model que permet avaluar el comportament de la recaiguda (model ADE). Els resultats obtinguts podrien permetre la identificació dels subjectes més vulnerables a la recaiguda en el consum d'etanol (experiment 1).

- Avaluar l'efecte de la NAC en la prevenció de la recaiguda en el consum d'etanol, en la població de rates Wistar vulnerables a la recaiguda (experiment 2).

- Explorar l'existència de possibles correlacions entre la vulnerabilitat a la recaiguda en el consum d'etanol i diferents alteracions bioquímiques tant durant el període d'abstinència tardana com després de la reintroducció en el consum d'etanol. Concretament s'han estudiat els nivells d'estrès oxidatiu i de diversos marcadors neuroinflamatoris (experiment 3).

- Analitzar el potencial mecanisme d'acció pel qual la NAC és capaç de prevenir la recaiguda en el consum d'etanol. En concret, s'aborden els seus efectes sobre l'estrès oxidatiu, l'estat neuroinflamatori i l'homeòstasi glutamatèrgica (experiment 4).

- Realitzar una exploració preliminar dels efectes de la NAC després d'una administració aguda sobre l'activació del SMCL i els mecanismes que subjacents en aquests efectes (experiment 5).

Resultats i discussió

Experiment 1. Identificació i caracterització de dues subpoblacions segons la seua vulnerabilitat a la recaiguda en el consum d'etanol.

Per a la realització de l'experiment 1 es van emprar dues cohorts de rates Wistar salvatges o “no seleccionades” que es van exposar al model ADE: experiment 1.A (43 mascles durant 32 setmanes) i experiment 1.B (38 femelles durant 26 setmanes). En aquest temps els animals van poder consumir etanol de manera voluntària a partir de botelles diferents (aigua, etanol al 5, 10 i 20% v/v). Aquests animals es van sotmetre a tres períodes d'abstinència a l'alcohol, aleatoris en el temps, en els quals únicament van tindre accés a l'aigua. Després de la reintroducció de les solucions d'etanol, al finalitzar cada període d'abstinència, es va determinar la **manifestació del fenomen ADE**. Per a això, es va comparar el consum d'etanol de cada animal (expressat en g/kg/dia) durant els tres últims dies abans del període d'abstinència i els tres dies després de la reintroducció de l'etanol. Quan el consum d'etanol havia augmentat per damunt del 50% del seu consum basal es va considerar que l'animal havia manifestat el fenomen ADE. Després de tres períodes d'abstinència els animals que van manifestar 2 o 3 fenòmens ADE es van catalogar com a animals més vulnerables a la recaiguda (**subpoblació ADE**) mentre que els animals que van manifestar 1 o cap fenomen ADE van ser assignats a la **subpoblació no-ADE**. Després de la classificació dels individus en dues subpoblacions es va procedir a la seua caracterització. Per a això es va analitzar el consum d'etanol, la preferència total d'etanol, així com la

preferència particular per cada solució d'etanol a la qual els animals van tindre accés.

Els resultats més rellevants de **l'experiment 1.A** van mostrar que:

- A partir de la cohort de 43 rates Wistar mascle, 30 animals es van assignar a la subpoblació ADE (70% d'animals) i 13 al subgrup no-ADE (30% d'animals).
- Els animals assignats a la subpoblació ADE van presentar tant un consum com una preferència total per l'etanol significativament inferiors al dels animals de la subpoblació no-ADE.
- Els animals ADE van mostrar una preferència significativament major per la dilució de 20% d'etanol que els animals no-ADE. Per contra, el grup no-ADE va manifestar una preferència significativament major per la dilució del 5% d'etanol que el grup ADE.

Els resultats més rellevants de **l'experiment 1.B** van demostrar que:

- A partir de la cohort de 38 rates Wistar femella, 22 animals es van classificar com a animals ADE (58% dels animals) i 16 com no-ADE (42% dels animals).
- La subpoblació ADE va mostrar una preferència total per l'etanol significativament inferior que la subpoblació no-ADE.
- La subpoblació ADE presentava una preferència significativament major per les dilucions més concentrades d'etanol (10 i 20% v/v) que la subpoblació no-ADE. Per contra, el grup no-ADE manifestava una preferència major per la dilució del 5% d'etanol que el grup ADE. Curiosament, sota les nostres

condicions experimentals, aquest comportament diferencial va tendir a desaparèixer a mesura que l'experiment es prolongava.

Els resultats d'aquest experiment han demostrat la utilitat del model ADE, emprant la rata Wistar no seleccionada, per a identificar als animals més vulnerables a manifestar un comportament de recaiguda. Tant en rates mascle com en rates femella, s'ha evidenciat que la major o menor predisposició a la recaiguda està associada amb uns trets fenotípics particulars. La identificació d'aquestes dues subpoblacions va ser de gran utilitat per al desenvolupament dels experiments posteriors: d'una banda en l'experiment 2 es va poder avaluar l'eficàcia anti-recaiguda de la NAC en animals amb una elevada predisposició a la recaiguda (ADE) i d'altra banda, en l'experiment 3 es va poder realitzar un estudi comparatiu de les potencials alteracions bioquímiques entre les dues subpoblacions amb la idea de poder correlacionar-les amb la vulnerabilitat dels animals a manifestar el comportament de recaiguda.

Experiment 2. Estudi de l'eficàcia de NAC en la prevenció de la recaiguda en consum d'etanol.

En aquest experiment es va tractar d'avaluar l'eficàcia de la NAC en la prevenció del comportament de la recaiguda en el consum d'alcohol en els individus més vulnerables. Per a això, es van emprar tant els animals mascle com les femelles prèviament caracteritzades com a subpoblacions ADE. En tots dos casos, els animals es van sotmetre a un nou període d'abstinència de 21 dies de duració, després del qual es van reintroduir les solucions d'alcohol durant 4 dies més. Durant els últims 10 dies d'abstinència i els 4 de reintroducció els animals van

rebre una injecció subcutània diària de NAC (total 14 dosi). En tots els animals tractats assignats als diferents grups experimentals es va avaluar la manifestació del fenomen ADE.

L'**experiment 2.A** es va dur a terme emprant 30 rates Wistar mascle (subpoblació ADE) dividides aleatòriament en 3 grups experimentals (n=10) El tractament rebut per cada grup va ser: vehicle, NAC 60 mg/kg o NAC 100 mg/kg. Els resultats obtinguts van revelar que:

- El grup que va rebre vehicle (grup control) va manifestar, com era esperable, el comportament de recaiguda.
- Els animals que van rebre NAC 60 mg/kg no sols no van manifestar el fenomen ADE, sinó que després de la reintroducció de l'alcohol, el seu consum va disminuir significativament respecte al seu consum basal
- El grup tractat amb NAC 100 mg/kg no va manifestar el fenomen ADE. En aquest grup experimental, el consum d'etanol durant la fase de reintroducció va ser similar al consum basal manifestat pels animals.

D'altra banda, l'**experiment 2.B** es va dur a terme amb 20 femelles (subpoblació ADE) assignades aleatòriament a 2 grups experimentals diferents (n=10), cadascun dels quals va rebre un tractament (vehicle o NAC 60 mg/kg). Els resultats van demostrar que:

- El grup tractat amb vehicle (control), curiosament, no va manifestar el fenomen ADE.
- El grup tractat amb NAC 60 mg/kg va mostrar una disminució significativa del consum d'etanol després de la reintroducció en comparació amb els seus valors basals.

Els resultats de l'experiment realitzat en mascles (**2.A.**) van reflectir que el tractament amb NAC presenta **una corba dosi-resposta amb forma de U invertida**, sent més eficaç la dosi de NAC 60 mg/kg que la dosi de NAC 100 mg/kg. Aquests resultats semblen estar d'acord amb els obtinguts per Kupchick i col·laboradors. Aquests autors, emprant procediments *in vitro*, van evidenciar que dosis baixes de NAC poden augmentar els nivells de glutamat extracel·lular, el qual activa els mGluR2/3 podent inhibir l'alliberament de glutamat sinàptic en nucli accumbens. No obstant això, dosis més elevades de NAC, les quals generaran nivells majors de glutamat extrasinàptic, també podrien activar els receptors mGluR5 a nivell postsinàptic, contrarestant així l'efecte inhibitori del receptor presinàptic (Kupchick et al., 2012). Aquest efecte dual en virtut de la dosi assajada de NAC s'ha tractat d'estudiar també en l'experiment 5 de la present tesi.

D'altra banda, els resultats obtinguts en l'**experiment 2.B** van impossibilitar l'avaluació de l'eficàcia anti-recaiguda de la NAC en femelles, ja que el grup control no va manifestar el ADE. De totes maneres, és innegable que, de nou, el tractament emprant una dosi de NAC 60 mg/kg va disminuir significativament el consum d'etanol després del període d'abstinència. Tal com s'ha manifestat amb anterioritat, en l'**experiment 1.B**, les diferències en les preferències d'etanol entre el grup ADE i no-ADE de femelles començaven a difuminar-se al final de l'estudi, la qual cosa podria estar anticipant l'absència de la manifestació del fenomen ADE observada en el grup control de l'experiment 2.B. Així doncs, a fi de tractar d'evitar aquestes limitacions en futurs experiments, s'hauria de considerar la utilització d'uns criteris més exigents en la classificació de les femelles com ADE

o no-ADE. Per eixample, es podria utilitzar una major grandària mostral o fins i tot acurtar la duració del procediment experimental.

En tot cas, els nostres resultats apunten en la mateixa direcció que altres estudis prèviament publicats per diversos grups. Així doncs, Lebourgeois i col·laboradors van observar que la NAC és capaç de bloquejar la represa del comportament d'autoadministració de la droga. No obstant això, es podria considerar que aquests experiments, en els quals l'animal només té accés a l'alcohol durant 15 o 45 minuts, tenen una validesa ecològica limitada. A més, aquests experiments només van permetre avaluar l'efecte agut de la NAC, ja que es va administrar en dosi única 1 hora abans de l'experiment (Lebourgeois et al., 2018, 2019). Pero altra banda, l'efecte del tractament crònic de la NAC ha sigut estudiat per altres grups. D'aquesta manera, Quintanilla i col·laboradors van mostrar que el tractament amb NAC 100 mg/kg durant 14 dies redueix el comportament del *binge drinking* (ingesta de una gran quantitat d'alcohol es un espai de temps molt curt) mesurat durant 60 minuts després d'un període d'abstinència (Quintanilla et al. 2018). Durant la realització d'aquesta tesi, aquest grup també va publicar que un tractament de NAC 40 mg/kg administrat durant 9 dies redueix el consum d'etanol durant una fase de reintroducció de 4 dies (Israel et al., 2021). Encara que aquest últim estudi s'assembla més al nostre disseny experimental, cal destacar que va ser dut a terme en femelles UChB, es a dir, en animals seleccionats per la seua alta preferència per l'etanol. No obstant això, el nostre estudi s'ha realitzat en rates Wistar seleccionades per manifestar un comportament recurrent de la recaiguda.

Experiment 3. Estat oxidatiu i neuroinflamatori en el cervell de rata Wistar mascle després de 21 dies d'abstinència a etanol. Efectes de la reintroducció d'etanol.

L'experiment 3 ha abordat l'estudi de les alteracions a nivell d'estrès oxidatiu (experiment 3.1) i de neuroinflamació (experiment 3.2) en diferents estadis del cicle de l'addicció de drogues (consum crònic, abstinència prolongada i re accés al consum d'etanol). Addicionalment, l'estudi s'ha dissenyat amb finalitats comparatives entre dues subpoblacions de rates diferents per la seua major o menor vulnerabilitat a la recaiguda: subpoblació ADE i no-ADE. En concret, en aquest experiment es van utilitzar els cervells de 10 rates mascle ADE (5 sacrificades després de 21 dies d'abstinència i 5 sacrificades després de 24 hores de re accés al consum d'etanol) i 13 rates mascle no-ADE (6 d'elles sacrificades després de 21 dies d'abstinència i 7 d'elles després de 24 hores de re accés al consum d'etanol). En el disseny d'aquest experiment es van incloure, a més, un grup d'animals que únicament havien consumit aigua (grup control; n=9) i un grup d'animals exposats de forma continuada al consum voluntari d'aigua i etanol emprant o el paradigma de 4 botelles (grup Etanol; n=6). Tots els animals pertanyents als diferents grups experimentals es van sacrificar per a poder extraure el seu encèfal. Tots els cervells es van conservar a -80 °C fins a la seua dissecció.

Per a la realització de l'**experiment 3.1** es va extraure el teixit de HIP i de AMG de cada animal d'experimentació. Aquests teixits, es van utilitzar per a mesurar els nivells de GSH i GSSG mitjançant

cromatografia líquida d'ultra alta resolució acoblada a una espectrometria de masses (UPLC-MS/MS). Els resultats més rellevants es van obtenir a nivell del HIP. Aquests resultats van mostrar que:

- L'estat oxidatiu determinat en el HIP dels animals exposats de forma continuada al consum d'etanol no es va veure alterat sota les nostres condicions experimentals.
- Després d'una abstinència de 21 dies es va identificar l'existència d'un ambient prooxidant en el HIP dels animals més vulnerables a la recaiguda (grup ADE). En concret, el quocient GSSG/GSH es va veure augmentat més d'un 200% respecte al grup control. Curiosament, aquesta alteració no es va detectar en els animals pertanyents al subgrup no-ADE.
- La reintroducció de 24 hores d'etanol va restaurar els nivells oxidatius alterats durant l'abstinència en el HIP del grup ADE.

En l'**experiment 3.2** es va utilitzar el teixit de la PFC per a determinar els nivells de mRNA de diferents mediadors inflamatoris (IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB i NLRP3) mitjançant la tècnica de RT-qPCR. Els resultats més rellevants de l'experiment 3.2 van mostrar:

- Un augment significatiu dels nivells de IL-1β en PFC durant la fase d'abstinència prolongada. Aquest increment es va identificar exclusivament en els animals del subgrup ADE.
- L'exposició crònica al consum d'etanol no va alterar cap dels paràmetres analitzats sota les nostres condicions experimentals.
- La reintroducció d'etanol va restaurar el increment detectat en els nivells de IL-1β durant la fase d'abstinència en el grup ADE.

- Els nivells de TNF-α van ser significativament més elevats en les rates ADE que en les no-ADE durant la fase d'abstinència.
- La reintroducció d'etanol va reduir els nivells elevats de TNF-α que s'havien determinat en les rates ADE després de l'abstinència.

En la literatura científica existeixen nombroses evidències que indiquen que el consum crònic d'etanol està associat amb un augment de l'estrès oxidatiu (Akhtar et al., 2017; Fernandes et al., 2017; Ramachandran et al., 2003; Uys, et al., 2014) i la neuroinflamació (Ezquer et al., 2019; Qin et al., 2009; Tiwari et al., 2009; Vallés et al., 2004). Una anàlisi detallada d'aquests articles mostra que, en la majoria d'ells, les taxes de consum d'etanol eren molt elevades, ja siga pel model d'exposició a l'etanol (ús de la dieta de Lieber i De Carli, model d'accés intermitent, etc) o per emprar rates seleccionades i criades per la seua elevada preferència o elevat consum de la droga. Per contra, en el nostre experiment es van utilitzar rates Wistar no seleccionades que van presentar un consum mitjà d'etanol de 2.21 ± 0.12 g/kg/dia, el qual és significativament menor que el dels articles citats amb anterioritat.

Cal també ressaltar que els resultats obtinguts en la present Tesi coincideixen, almenys en part, amb els publicats per Jung i col·laboradors en 2016. En concret, aquest grup estava estudiant les diferències, a nivell oxidatiu, en el HIP i el cerebel de rates mascle i femella després de 3 setmanes d'abstinència al etanol (Jung & Metzger, 2016). Els nostres resultats van en la mateixa línia que les seues troballes, ja que en les seues condicions experimentals també van detectar un augment de l'estrès oxidatiu en HIP de les rates. No

obstant això, cal destacar que els nostres resultats han revelat per primera vegada una **correlació entre el comportament de recaiguda i l'existència d'alteracions en l'estrès oxidatiu i/o la neuroinflamació durant la fase d'abstinència tardana.**

En relació amb les alteracions determinades en els mediadors inflamatoris durant la fase d'abstinència, els estudis publicats no permeten arribar a una conclusió ferma. D'una banda, alguns estudis van trobar condicions proinflamatòries durant la fase d'abstinència En relació amb les alteracions determinades en els mediadors inflamatoris durant la fase d'abstinència, els estudis publicats no permeten arribar a una conclusió ferma. D'una banda, alguns estudis van trobar condicions pro inflamatòries durant la fase d'abstinència primerenca (Knapp et al., 2016; Schneider et al., 2017). No obstant això, altres autors han indicat que el cessament del consum d'etanol restaura els nivells de mediadors inflamatoris (Whitman et al., 2013). En relació a l'estudi durant un període prolongat d'abstinència, d'una banda, Vetreno i col·laboradors van detectar nivells elevats de TNF-α en rates després de 25 dies d'abstinència a l'etanol (Vetreno et al., 2013), mentre que un altre estudi realitzat 28 dies després del cessament del consum d'etanol, no va detectar alteracions en les citocines del còrtex frontal però sí en el marcador d'activació microglial Iba-1 (Sanchez-Alavez et al., 2019).

Sorprenentment, tant en l'**experiment 3.1** com l'**experiment 3.2** de la present tesi, el re accés al consum d'etanol (24 hores) després d'una abstinència prolongada és capaç d'alleujar les alteracions trobades en rates abstinents amb tendència a la recaiguda. Per contra, l'estudi de Cuitavi i col·laboradors, dut a terme amb rates Sprague-Dawley

femelles, va determinar que els nivells de IL-1β en PFC van augmentar després de 5 dies de reintroducció al consum d'etanol, en comparació amb els nivells mesurats en el període d'abstinència. No obstant això, s'ha de considerar que, a contrari que els nostres animals ADE, aquests animals no van manifestar un comportament de recaiguda després del període d'abstinència. En general, els nostres resultats suggereixen que l'estrès oxidatiu i la senyalització pro-inflamatòria pot tindre un paper clau en el procés de la recaiguda en el consum d'etanol.

Experiment 4. Desentranyant el mecanisme d'acció subjacent en l'efecte anti-recaiguda de la NAC.

Després de demostrar que la NAC té un efecte anti-recaiguda en el consum d'etanol i detectar l'existència d'alteracions inflamatòries i prooxidants relacionades amb la vulnerabilitat a la recaiguda, en la present tesi es va decidir estudiar si la NAC podria actuar a nivell d'aquestes alteracions donant una explicació mecanística al seu efecte per a prevenir les recaigudes després d'un període d'abstinència. De la mateixa manera i atès que a la NAC també se li atribueixen propietats glutamatèrgiques, es va estudiar el seu efecte sobre l'expressió proteica de GLT1, GLAST i xCT, coneguts transportadors del glutamat, en el STM. Amb aquesta finalitat, 15 animals de la subpoblació ADE es van sotmetre a un nou període d'abstinència de 21 dies en el qual van rebre, mitjançant una injecció subcutània, els diferents tractaments assajats (vehicle, NAC 60 mg/kg o NAC 100 mg/kg; n=5 per grup). Cada tractament es va administrar una vegada al dia durant 10 dies. Tots els animals es van sacrificar durant la fase d'abstinència i sempre 24 hores després de l'última dosi administrada.

Els cervells extrets es van disseccionar per a així obtenir els diferents nuclis cerebrals de interès. Així doncs, es va utilitzar el teixit de HIP i de AMG per a estudiar els nivells d'estrès oxidatiu mitjançant UPLC-MS/MS en l'**experiment 4.1**. Els resultats obtinguts més rellevants van ser:

- Durant la fase d'abstinència prolongada, tant els nivells de GSSG com el quocient GSSG/GSH estan augmentats de manera significativa en el HIP de les rates més vulnerables a la recaiguda (subpoblació ADE).
- El tractament amb NAC 60 mg/kg o amb NAC 100 mg/kg restaura els paràmetres alterats. I, sorprenentment, en cap cas els nivells de GSH es veuen incrementats.
- En la AMG no es detecten canvis significatius de l'estat oxidatiu sota cap condició experimental.

En **l'experiment 4.2** es va emprar el teixit procedent de la PFC per a avaluar els nivells d'ARN missatger de diversos mediadors inflamatoris (IL-1β, IL-6, TNF-α, HMGB1, iNOS, NfκB i NLRP3) mitjançant RT-qPCR. Els resultats van mostrar que:

- Els nivells de IL-1β i TNF-α havien augmentat significativament durant el període d'abstinència tardana.
- El tractament amb NAC 100 mg/kg va ser capaç de reduir de manera significativa els nivells de TNF-α.

Finalment, en **l'experiment 4.3** es va estudiar en dues regions del STM (estriat dorsolateral (DS) i nucli accumbens) els nivells d'expressió de GLT1, GLAST i xCT mitjançant la tècnica de Western Blot. Els resultats més destacables van ser:

- El consum continuat d'etanol sota les nostres condicions experimentals no modifica els nivells d'expressió de cap de les proteïnes estudiades.
- Durant la fase d'abstinència, els nivells de GLT1 i GLAST estaven significativament reduïts en el nucli accumbens de les nostres rates.
- El tractament amb NAC 60 mg/kg o 100 mg/kg no va induir cap canvi sobre l'expressió de GLT1, GLAST i xCT durant la fase d'abstinència.
- Cap de les proteïnes analitzades es va veure alterades sota cap de les condicions experimentals en el DS.

Els nostres resultats suggereixen que **les propietats antioxidants i antiinflamatòries de la NAC semblen explicar, almenys parcialment, el seu efecte anti-recaiguda en el consum d'etanol.** A pesar que nombrosos estudis han demostrat les propietats antioxidants i antiinflamatòries de la NAC després de l'exposició dels subjectes experimentals al consum d'etanol (Mocelin et al., 2018; Quintanilla et al., 2018; Israel et al., 2021), els treballs dissenyats per a estudiar el seu efecte durant el període d'abstinència són escassos. Els nostres resultats mostren com la NAC és capaç de revertir els desequilibris oxidatius associats amb un període prolongat d'abstinència. En aquesta mateixa línia, Mocelin i col·laboradors van demostrar en 2019 que el tractament amb NAC és capaç de disminuir els nivells d’estrès oxidatiu detectats en el peix zebra després de 24 hores d'abstinència. En relació al seu efecte antiinflamatori, en les nostres condicions experimentals NAC 100 mg/kg va ser capaç de reduir els nivells de TNF-α en PFC durant la fase d'abstinència tardana. Igualment, un

treball previ va suggerir que el tractament amb NAC 60 mg/kg o 90 mg/kg és capaç de reduir en el HIP i còrtex frontal de la rata els nivells d'uns certs mediadors inflamatoris augmentats després de 24 hores d'abstinència a l'etanol (Schenider et al., 2017).

Finalment, en aquest bloc d'experiments també es van avaluar els efectes que el consum continuat d'etanol o l'experimentació d'un estat d'abstinència prolongat poden tindre sobre la senyalització glutamatèrgica en dues regions diferents del STM (**experiment 4.3**). Els resultats més remarcables es van obtenir en el nucli accumbens. Concretament, el consum continuat d'etanol sota les nostres condicions experimentals no va modificar de manera significativa l'expressió proteica de GLT1, GLAST ni xCT. A priori, aquests resultats s'allunyen dels descrits en la literatura, ja que existeixen nombroses evidèncíes d'una disminució de GLT1 en el nucli accumbens de rates P mascle quan són exposades al consum crònic d'etanol (Alhaddad et al., 2014; Das et al., 2015; Hakami et al., 2016; Sari et al., 2013). Com s'ha comentat amb anterioritat, les taxes de consum d'etanol per part de les nostres rates Wistar salvatges van ser significativament menors que les de les rates P emprades per altres grups. Probablement, aquesta diferència és l'explicació més plausible a aquesta absència d'efectes. No obstant això, curiosament, els nostres resultats mostren que, després de 3 setmanes d'abstinència, els nivells de GLT1 i GLAST en nucli accumbens es troben reduïts. Una vegada més, les investigacions existents focalitzades en l'estudi dels efectes induïts per un període d'abstinència més o menys prolongat són escassos. Els pocs resultats disponibles en la bibliografia, indiquen que l'abstinència primerenca en rates mascle no afecta als nivells d'expressió de GLT1 i

GLAST (Meléndez et al., 2005; Lebourgeois et al., 2019) però, en canvi, sí que redueix els nivells de xCT en nucli accumbens (Peanya et al., 2014; Lebourgeois et al., 2019). Un únic treball ha explorat l'efecte de dues setmanes d'abstinència sobre l'expressió d'aquestes proteïnes en el nucli accumbens de rates P femelles i ha reportat una disminució en els nivells de GLAST però cap canvi en GLT1 i xCT (Ding et al., 2013). Per tant, el nostre treball demostra per primera vegada que una abstinència prolongada a l'etanol és capaç d'induir una reducció en l'expressió total de proteïna GLT1 i GLAST sense afectar la quantitat de proteïna de xCT en el nucli accumbens.

Sorprenentment, el tractament amb NAC durant la fase d'abstinència no va modificar de manera significativa els nivells de cap de les proteïnes alterades. No té cap efecte sobre l'expressió de les proteïnes estudiades en les nostres condicions experimentals. Així doncs, els nostres resultats no coincideixen amb els descrits per Lebourgeois i col·laboradors, els qui van publicar que la NAC disminueix l'expressió de GLT1 en nucli accumbens en animals sotmesos a un protocol d'autoadministració operant d'etanol (Lebourgeois et al., 2019). Finalment, caldria pensar que l'efecte anti-recaiguda de la NAC podria deure's a canvis en l'activitat d'aquestes proteïnes i no sols al nivell d’expressió total d'aquestes. Per tot això, serien necessaris experiments addicionals per a poder avaluar aquesta hipòtesis de treball.

Experiment 5. Efectes aguts de la NAC en l'activació del sistema mesocorticolímbic

Aquest conjunt d'experiments es van dissenyar per a aprofundir en els efectes aguts que poden produir diferents dosis de NAC sobre l'activació del SMCL. Per a això en l'experiment 5.1 es van analitzar els efectes aguts que diferents dosis sistèmiques de NAC (30 mg/kg o 120 mg/kg) poden tindre en l'activació de les neurones del SMCL. Aquest efecte es va determinar a través de la quantificació de cèl·lules cFOS inmunorreactivas (cFOS-IR) en el nucli accumbens. A més, amb l'objectiu d'aprofundir en el mecanisme subjacent a l'activació, es va avaluar l'efecte de la presència d'un modulador negatiu al·lostèric de mGluR5 com és el **MTEP**. En aquest experiment es van emprar 34 rates Wistar mascle que van rebre dues injeccions intraperitoneals administrades de manera consecutiva dues hores abans de ser sacrificades per a poder extraure el seu encèfal. La primera injecció va consistir en l'administració d'una dosi de salí o MTEP (0.1 mg/kg) i la segona injecció va consistir en l'administració de vehicle, NAC 30 mg/kg o NAC 120 mg/kg. De la combinació de les dues injeccions van sorgir 6 grups experimentals (n=6, excepte el grup salí/vehicle n=4). A partir dels cervells extrets es van obtenir seccions de 40 µM de grossària amb ajuda d'un microtom de congelació. A continuació, l'aplicació de procediments immunohistoquímics va permetre la quantificació de cèl·lules cFOS-IR en nucli accumbens. Els resultats més rellevants de l'experiment van revelar que:

- L'administració sistèmica d'una dosi de NAC 120 mg/kg va incrementar de manera significativa el nombre de cèl·lules

cFOS-IR en el nucli accumbens. La presència de MTEP (0.1 mg/kg) va bloquejar l'augment de CFOS-IR induït per una dosi de NAC 120 mg/kg.

A tenor dels nostres resultats, una dosi baixa de NAC no és capaç d'activar el SMCL. Per contra, sota les nostres condicions experimentals, hem observat que dosis altes de NAC poden causar una activació del SMCL en nucli accumbens, que es anul·lada per l'administració de MTEP. Aquests resultats van coincidir amb les observacions descrites per Kupchick i col·laboradors en 2012. Concretament, aquests autors van demostrar, emprant procediments d'electrofisiologia *in vitro*, que l'activació de NAC sobre el SMCL depèn de la dosi administrada. Segons aquests autors això es deu al fet de que l'administració d'una dosi baixa de NAC, a través del sistema xC-, causa un augment moderat del glutamat extrasinàptic que al seu torn activa el receptor presinàptic mGluR2/3 i inhibeix la sinapsi glutamatèrgica. Contràriament, dosis elevades de NAC estimulen la alliberació d'uns nivells majors de glutamat extrasinàptic que també activen el receptor postsinàptic mGluR5, el qual, al seu torn, acaba activant la neurona postsinàptica (Kupchick et al., 2012). Així doncs, els nostres resultats recolzen els mecanismes proposats amb anterioritat. Igualment, cal assenyalar que un estudi recent que emprava el paradigma de la preferència de lloc condicionada va mostrar que l'administració de NAC té propietats reforçadores quan és administrada a raó de 120 mg/kg però no quan s'injecten 60 mg/kg (Laverde et al., 2021). Aquest estudi, igual que els nostres resultats, suggereix que només les dosis més elevades de NAC són capaces d'activar el SMCL.

D'altra banda, en l'**experiment 5.2** es van explorar els efectes de l'administració d'una dosi elevada de NAC (120 mg/kg) sobre els efectes reforçadors induïts per l'etanol per se. Per a això es va analitzar l'activitat locomotora mostrada pels animals d'experimentació, així com els nivells de cFOS-IR després d'una microinjecció de 150 nmol d'etanol en l'àrea ventral tegmental (VTA) en presència o absència de NAC. En aquest experiment es va haver d'implantar una cànula intra-VTA a 24 rates Wistar mascle. El dia de l'experiment van rebre una dosi de salí o NAC 120 mg/kg mitja hora abans que se'ls administrés intra-VTA o be líquid cerebroespinal artificial (aCSF) o bé 150 nmol d'etanol. En primer lloc, es va determinar la distància recorreguda per cada animal en un camp obert durant 20 minuts. Una vegada transcorregudes 2 hores des de la injecció intra-VTA els animals es van sacrificar. Els cervells perfosos es van seccionar amb ajuda d'un microtom de congelació i mitjançant procediments immunohistoquímics, es va quantificar l'expressió de cèl·lules cFOS-IR en el nucli accumbens. Els resultats van mostrar que:

- La variació en l'activitat locomotora determinada en cada grup experimental no va presentar canvis significatius encara que va evidenciar que un augment de la distància recorreguda quan es van administrar bé 150 nmol d'etanol o bé 120 mg/kg de NAC.
- L'administració sistèmica d'una dosi de NAC 120 mg/kg, va induir un augment significatiu en l'expressió de cèl·lules cFOS-IR en el nucli accumbens, de manera semblant al que es va observar en el cas de l'activitat locomotora.

- La -injecció intra-VTA de 150 nmol d'etanol també va incrementar el nombre de cèl·lules cFOS-IR.
- Sorprenentment, quan es van co-administrar NAC i etanol el nombre de cèl·lules cFOS-IR no va presentar canvis significatius. A més, l'activitat locomotora també es va reduir, encara que de forma no significativa, respecte a la determinada després de l'administració de NAC o etanol de forma aïllada.

Com s'ha comentat abans, en el nostre experiment 5.1. hem observat com l'administració de NAC 120 mg/kg o de 150 nmol d'etanol augmenta el nombre de cèl·lules cFOS-IR en nucli accumbens, és a dir, té efectes activadors sobre el SMCL. Curiosament, quan s'administren totes dues substàncies al mateix temps aquests efectes no es manifesten. Laverde i col·laboradors van observar de manera similar en els seus experiments que, tant l'administració de NAC 120 mg/kg com la d'etanol van induir per se condicionament preferent de lloc. No obstant això, quan totes dues substàncies es van administrar conjuntament junts durant la fase de condicionament, els efectes reforçadors de totes dues substàncies van desaparèixer (Laverde et al., 2021). Davant els nostres resultats i els descrits en la bibliografia es podria hipotetitzar que totes dues molècules podrien interaccionar entre si anul·lant-se els efectes que ambdues produeixen per separat. Una explicació plausible d'aquest fenomen podria estar mediat pel segrest del ACD per part de la NAC. En la literatura s'han descrit àmpliament els efectes activadors de l'administració d'etanol. En aquesta línia, molts autors han demostrat que part dels efectes activadors de l'etanol estan mediats pel ACD, el seu primer metabòlit

(Foddai et al., 2004; Melis et al., 2007). El ACD és una molècula altament reactiva que podria ser segrestada per molècules amb ponts disulfur com la D-Penicilamina o la L-cisteïna, que al seu torn han demostrat reduir el consum d'etanol en models preclínics (Peanya et al., 2009, 2010, 2013; Orrico et al., 2013; Martí-Prats et al., 2015). Curiosament, la NAC és un precursor de la L-cisteïna, per la qual cosa seria viable sospitar que la seua administració provoqués el segrest del ACD generat de manera local en el VTA després de l'administració d'etanol. De totes maneres, és necessari dur a terme més experiments per a corroborar aquesta hipòtesi i comprovar si aquest potencial mecanisme d'acció de la NAC també està relacionat amb el seu efecte anti-recaiguda.

Conclusions

- El model preclínic ADE ha permés identificar diferents subpoblacions de rates mascle Wistar que difereixen en la seua vulnerabilitat a la recaiguda en el consum d'etanol. A més, cada subpoblació va presentar patrons diferents de consum i de preferència pel de etanol. Per altra banda, segons els resultats obtinguts, els criteris per a categoritzar les rates Wistar femella han de ser refinats.

- NAC inhibeix el comportament de la recaiguda en el consum d'etanol manifestat per rates Wistar vulnerables a la recaiguda. Els millors resultats es van obtenir després de l'administració de varies dosis de NAC 60 mg/kg.

- Després d'un període d'abstinència prolongada, el HIP va presentar condiciones prooxidants i també es van detectar processos neuroinflamatoris en PFC. Aquests efectes es van observar únicament en els animals més vulnerables a la recaiguda en el consum d'etanol.

- Els nivells de GLT1 i GLAST en el nucli accumbens estan significativament reduïts durant la fase d'abstinència tardana en els animals vulnerables a la recaiguda.

- La represa del consum d'etanol després d'un període d'abstinència alleuja les alteracions bioquímiques detectades durant la fase d'abstinència en animals vulnerables a la recaiguda.

- El tractament amb NAC durant la fase d'abstinència restaura els nivells oxidatius de HIP i els nivells de TNF-α en PFC, la qual cosa suggereix que les seues capacitats antioxidants i antiinflamatòries estan relacionades, almenys en part, amb el seu efecte anti-recaiguda.

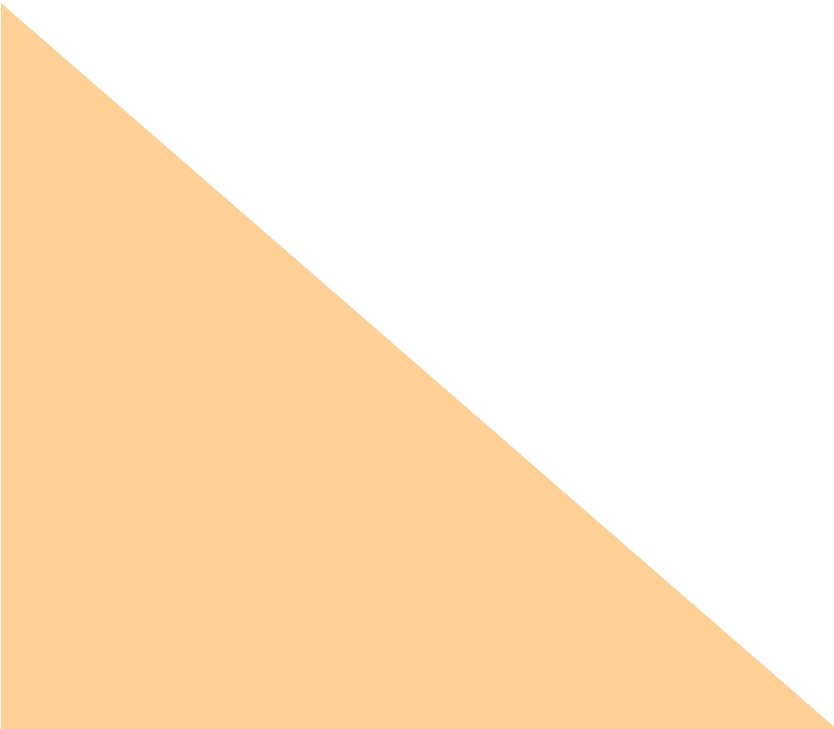
- NAC no afecta a l'expressió de GLT1, GLAST i xCT durant un període d'abstinència prolongada en el STM d'animals vulnerables a la recaiguda.

- El tractament agut amb NAC té un efecte dosi-dependent en la transmissió glutamatèrgica. Dosis elevades de NAC activen el SMCL, reflectit a través d'un augment en l'expressió de cèl·lules

cFOS-IR en el nucli accumbens. El modulador negatiu al·lostèric de mGluR5 (MTEP) és capaç de revertir aquest efecte, la qual cosa suggereix que els efectes activadors de NAC estan mediats per mGluR5.

- L'augment de cèl·lules cFOS-IR en nucli accumbens induït per l'etanol és atenuat quan es co-administra en presència d'una dosi elevada de NAC.

9. BIBLIOGRAPHY



Abernathy, K., Chandler, L. J., & Woodward, J. J. (2010). Alcohol and the prefrontal cortex. *International Review of Neurobiology*, *91*(C), 289–320. https://doi.org/10.1016/S0074-7742(10)91009-X

Abulseoud, O. A., Camsari, U. M., Ruby, C. L., Kasasbeh, A., Choi, S., & Choi, D. S. (2014). Attenuation of ethanol withdrawal by ceftriaxone-induced upregulation of glutamate transporter EAAT2. *Neuropsychopharmacology*, *39*(7), 1674–1684. https://doi.org/10.1038/npp.2014.14

Agabio, R., Carai, M. A. M., Lobina, C., Pani, M., Reali, R., Vacca, G., Gessa, G. L., & Colombo, G. (2000). Development of short-lasting alcohol deprivation effect in sardinian alcohol-preferring rats. *Alcohol (Fayetteville, N.Y.)*, *21*(1), 59–62. https://doi.org/10.1016/S0741-8329(00)00072-0

Agabio, R., & Leggio, L. (2018). Baclofen in the Treatment of Patients With Alcohol Use Disorder and Other Mental Health Disorders. *Frontiers in Psychiatry*, *9*, *464*. https://doi.org/10.3389/FPSYT.2018.00464

Akhtar, F., Rouse, C. A., Catano, G., Montalvo, M., Ullevig, S. L., Asmis, R., Kharbanda, K., & Maffi, S. K. (2017). Acute maternal oxidant exposure causes susceptibility of the fetal brain to inflammation and oxidative stress. *Journal of Neuroinflammation*, *14*(1), 1-17. https://doi.org/10.1186/S12974-017-0965-8

Alasmari, F., Goodwani, S., McCullumsmith, R. E., & Sari, Y. (2018). Role of glutamatergic system and mesocorticolimbic circuits in alcohol dependence. *Progress in Neurobiology*, *171*, 32–49. https://doi.org/10.1016/j.pneurobio.2018.10.001

Aldini, G., Altomare, A., Baron, G., Vistoli, G., Carini, M., Borsani, L., & Sergio, F. (2018). N-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radical Research*, *52*(7), 751–762. https://doi.org/10.1080/10715762.2018.1468564

Alfonso-Loeches, S., Ureña-Peralta, J. R., Morillo-Bargues, M. J., la Cruz, J. O. de, & Guerri, C. (2014). Role of mitochondria ROS generation in ethanol-induced NLRP3 inflammasome activation and cell

death in astroglial cells. *Frontiers in Cellular Neuroscience*, *8*, 216. https://doi.org/10.3389/fncel.2014.00216

Alhaddad, H., Kim, N. T., Aal-Aaboda, M., Althobaiti, Y. S., Leighton, J., Boddu, S. H. S., Wei, Y., & Sari, Y. (2014). Effects of MS-153 on chronic ethanol consumption and GLT1 modulation of glutamate levels in male alcohol-preferring rats. *Frontiers in Behavioral Neuroscience*, *8*, 366. https://doi.org/10.3389/fnbeh.2014.00366

Almansa, I., Barcia, J. M., López-Pedrajas, R., Muriach, M., Miranda, M., & Romero, F. J. (2013). Naltrexone reverses ethanol-induced rat hippocampal and serum oxidative damage. *Oxidative Medicine and Cellular Longevity*, *2013*. https://doi.org/10.1155/2013/296898

Amaral, V. C. S., Morais-Silva, G., Laverde, C. F., & Marin, M. T. (2021). Susceptibility to extinction and reinstatement of ethanol-induced conditioned place preference is related to differences in astrocyte cystine-glutamate antiporter content. *Neuroscience Research*, *170*, 245–254. https://doi.org/10.1016/j.neures.2020.07.002

Amen, S. L., Piacentine, L. B., Ahmad, M. E., Li, S. J., Mantsch, J. R., Risinger, R. C., & Baker, D. A. (2011). Repeated n-acetyl cysteine reduces cocaine seeking in rodents and craving in cocaine-dependent humans. *Neuropsychopharmacology*, *36*(4), 871–878. https://doi.org/10.1038/npp.2010.226

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5th ed.). https://doi.org/10.1176/appi.books.9780890425596

Aoyama, K. (2021). Glutathione in the brain. *International Journal of Molecular Sciences,* 22(9), 5010. https://doi.org/10.3390/ijms22095010

Asztely, F., Erdemli, G., & Kullmann, D. M. (1997). Extrasynaptic Glutamate Spillover in the Hippocampus: Dependence on Temperature and the Role of Active Glutamate Uptake. *Neuron, 18*(2), 281-293. http://doi.org/10.1016/s0896-6273(00)80268-8

Bachteler, D., Economidou, D., Danysz, W., Ciccocioppo, R., & Spanagel, R. (2005). The effects of acamprosate and neramexane on cue-induced reinstatement of ethanol-seeking behavior in rat. *Neuropsychopharmacology*, *30*(6), 1104–1110. https://doi.org/10.1038/SJ.NPP.1300657

Back, S. E., McCauley, J. L., Korte, K. J., Gros, D. F., Leavitt, V., Gray, K. M., Hamner, M. B., DeSantis, S. M., Malcolm, R., Brady, K. T., & Kalivas, P. W. (2016). A double-blind, randomized, controlled pilot trial of N-acetylcysteine in veterans with posttraumatic stress disorder and substance use disorders. *Journal of Clinical Psychiatry*, *77*(11), e1439–e1446. https://doi.org/10.4088/JCP.15m10239

Badisa, R. B., Goodman, C. B., & Fitch-Pye, C. A. (2013). Attenuating effect of N-acetyl-L-cysteine against acute cocaine toxicity in rat C6 astroglial cells. *International Journal of Molecular Medicine*, *32*(2), 497–502. https://doi.org/10.3892/IJMM.2013.1391

Baker, D. A., McFarland, K., Lake, R. W., Shen, H., Toda, S., & Kalivas, P. W. (2003). N-acetyl cysteine-induced blockade of cocaine-induced reinstatement. *Annals of the New York Academy of Sciences*, *1003*, 349–351. https://doi.org/10.1196/ANNALS.1300.023

Baker, D. A., Xi, Z. X., Shen, H., Swanson, C. J., & Kalivas, P. W. (2002). The Origin and Neuronal Function of In Vivo Nonsynaptic Glutamate. *Journal of Neuroscience*, *22*(20), 9134–9141. https://doi.org/10.1523/JNEUROSCI.22-20-09134.2002

Banks, W. A. (2015). The blood-brain barrier in neuroimmunology: Tales of separation and assimilation. *Brain, Behavior, and Immunity*, *44*, 1–8. https://doi.org/10.1016/J.BBI.2014.08.007

Bardgett, M. E., & Henry, J. D. (1999). Locomotor activity and accumbens Fos expression driven by ventral hippocampal stimulation require D1 and D2 receptors. *Neuroscience*, *94*(1), 59–70. https://doi.org/10.1016/S0306-4522(99)00303-6

Barrick, C., & Connors, G. J. (2002). Relapse prevention and maintaining abstinence in older adults with alcohol-use disorders.

Drugs and Aging, *19*(8), 583–594. https://doi.org/10.2165/00002512-200219080-00004

Baxter-Potter, L. N., Henricks, A. M., Berger, A. L., Bieniasz, K. v., Lugo, J. M., & McLaughlin, R. J. (2017). Alcohol vapor exposure differentially impacts mesocorticolimbic cytokine expression in a sex-, region-, and duration-specific manner. *Neuroscience*, *346*, 238–246. https://doi.org/10.1016/j.neuroscience.2017.01.015

Becker, H. (2009). Alcohol Dependence, Withdrawal and Relapse. *Alcohol research & health, 31*(4), 348-361.

Belin, D., Belin-Rauscent, A., Murray, J. E., & Everitt, B. J. (2013). Addiction: failure of control over maladaptive incentive habits. *Current Opinion in Neurobiology*, *23*(4), 564–572. https://doi.org/10.1016/J.CONB.2013.01.025

Belin, D., & Everitt, B. J. (2008). Cocaine Seeking Habits Depend upon Dopamine-Dependent Serial Connectivity Linking the Ventral with the Dorsal Striatum. *Neuron*, *57*(3), 432–441. https://doi.org/10.1016/J.NEURON.2007.12.019

Belin-Rauscent, A., Fouyssac, M., Bonci, A., & Belin, D. (2016). How Preclinical Models Evolved to Resemble the Diagnostic Criteria of Drug Addiction. *Biological Psychiatry*, *79*(1), 39–46. https://doi.org/10.1016/J.BIOPSYCH.2015.01.004

Bell, R. L., Hauser, S. R., Liang, T., Sari, Y., Maldonado-Devincci, A., & Rodd, Z. A. (2017). Rat animal models for screening medications to treat alcohol use disorders. *Neuropharmacology*, *122*, 201–243. https://doi.org/10.1016/J.NEUROPHARM.2017.02.004

Bell, R. L., Rodd, Z. A., Lumeng, L., Murphy, J. M., & McBride, W. J. (2006). The alcohol-preferring P rat and animal models of excessive alcohol drinking. *Addiction Biology*, *11*(3–4), 270–288. https://doi.org/10.1111/J.1369-1600.2005.00029.X

Bell, R. L., Sable, H. J. K., Colombo, G., Hyytia, P., Rodd, Z. A., & Lumeng, L. (2012). Animal models for medications development targeting alcohol abuse using selectively bred rat lines: neurobiological and pharmacological validity. *Pharmacology, Biochemistry, and*

Behavior, *103*(1), 119–155. https://doi.org/10.1016/J.PBB.2012.07.007

Berríos-Cárcamo, P., Quezada, M., Quintanilla, M. E., Morales, P., Ezquer, M., Herrera-Marschitz, M., Israel, Y., & Ezquer, F. (2020). Oxidative Stress and Neuroinflammation as a Pivot in Drug Abuse. A Focus on the Therapeutic Potential of Antioxidant and Anti-Inflammatory Agents and Biomolecules. *Antioxidants (Basel, Switzerland)*, *9*(9), 1–26. https://doi.org/10.3390/ANTIOX9090830

Blanco, A. M., & Guerri, C. (2007). Ethanol intake enhances inflammatory mediators in brain: role of glial cells and TLR4/IL-1RI receptors. *Frontiers in Bioscience*, *12*(7), 2616-2630. http://doi.org/10.2741/2259

Bradlow, R. C., Berk, M., Kalivas, P. W., Back, S. E., & Kanaan, R. A. (2022). The Potential of N-Acet³yl-L-Cysteine (NAC) in the Treatment of Psychiatric Disorders. *CNS drugs*, 36 (5), 451-482. http://doi.org/10.1007/s40263-022-00907-3

Brebner, K., Wong, T. P., Liu, L., Liu, Y., Campsall, P., Gray, S., Phelps, L., Phillips, A. G., & Wang, Y. T. (2005). Nucleus accumbens long-term depression and the expression of behavioral sensitization. *Science (New York, N.Y.)*, *310*(5752), 1340–1343. https://doi.org/10.1126/SCIENCE.1116894

Brieger, K., Schiavone, S., Miller, F. J., & Krause, K. H. (2012). Reactive oxygen species: From health to disease. In *Swiss Medical Weekly 142*, w13659. https://doi.org/10.4414/smw.2012.13659

Brousse, G., Arnaud, B., Vorspan, F., Richard, D., Dissard, A., Dubois, M., Pic, D., Geneste, J., Xavier, L., Authier, N., Sapin, V., Llorca, P.-M., de Chazeron, I., Minet-Quinard, R., & Schmidt, J. (2012). Alteration of Glutamate/GABA Balance During Acute Alcohol Withdrawal in Emergency Department: A Prospective Analysis. *Alcohol and alcoholism,* *47*(5), 501-508. https://doi.org/10.1093/alcalc/ags078

Brown, R. M., Kupchik, Y. M., & Kalivas, P. W. (2013). The story of glutamate in drug addiction and of N-acetylcysteine as a potential

pharmacotherapy. *JAMA Psychiatry, 70*(9), 895–897. https://doi.org/10.1001/jamapsychiatry.2013.2207

Burish, T. G., Maisto, S. A., Cooper, A. M., & Sobell, M. B. (1981). Effects of voluntary short-term abstinence from alcohol on subsequent drinking patterns of college students. *Journal of Studies on Alcohol*, *42*(11), 1013–1020. https://doi.org/10.15288/JSA.1981.42.1013

Campos-Jurado, Y., Igual-López, M., Padilla, F., Zornoza, T., Granero, L., Polache, A., Agustín-Pavón, C., & Hipólito, L. (2019). Activation of MORs in the VTA induces changes on cFos expression in different projecting regions: Effect of inflammatory pain. *Neurochemistry International*, *131*, 104521. https://doi.org/10.1016/J.NEUINT.2019.104521

Cannella, N., Ubaldi, M., Masi, A., Bramucci, M., Roberto, M., Bifone, A., & Ciccocioppo, R. (2019). Building better strategies to develop new medications in Alcohol Use Disorder: Learning from past success and failure to shape a brighter future. *Neuroscience and Biobehavioral Reviews*, *103*, 384–398. https://doi.org/10.1016/J.NEUBIOREV.2019.05.014

Cano-Cebrián, M. J., Zornoza-Sabina, T., Guerri, C., Polache, A., & Granero, L. (2003). Local acamprosate modulates dopamine release in the rat nucleus accumbens through NMDA receptors: an in vivo microdialysis study. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *367*(2), 119–125. https://doi.org/10.1007/S00210-002-0674-3

Carnicella, S., Ron, D., & Barak, S. (2014). Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. *Alcohol*, 48(3), 243-252. http://doi.org/10.1016/j.alcohol.2014.01.006

Cekanaviciute, E., & Buckwalter, M. S. (2016). Astrocytes: integrative regulators of neuroinflammation in stroke and other neurological diseases. *Neurotherapeutics*, 13(4), 685-701. http://doi.org/10.1007/s13311-016-0477-8

Centonze, D., Picconi, B., Gubellini, P., Bernardi, G., & Calabresi, P. (2001). Dopaminergic control of synaptic plasticity in the dorsal

striatum. *The European Journal of Neuroscience*, *13*(6), 1071–1077. https://doi.org/10.1046/J.0953-816X.2001.01485.X

Cepeda, C., Buchwald, N. A., & Levine, M. S. (1993). Neuromodulatory actions of dopamine in the neostriatum are dependent upon the excitatory amino acid receptor subtypes activated. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(20), 9576–9580. https://doi.org/10.1073/PNAS.90.20.9576

Chaudhry, F. A., Lehre, K. P., Van, M., Campagne, L., Ottersen, O. P., Danbolt, N. C., & Storm-Mathisen, J. (1995). Glutamate Transporters in Glial Plasma Membranes: Highly Differentiated Localizations Revealed by Quantitative Ultrastructural Immunocytochemistry. *Neuron* *15*(3), 711-720. http://doi.org/10.1016/0896-6273(95)90158-2

Ch'Ng, S. S., & Lawrence, A. J. (2018). Investigational drugs for alcohol use disorders: a review of preclinical data. In *Expert Opinion on Investigational Drugs* *27*(5), 459–474. https://doi.org/10.1080/13543784.2018.1472763

Clergue-Duval, V., Coulbault, L., Questel, F., Cabé, N., Laniepce, A., Delage, C., Boudehent, C., Bloch, V., Segobin, S., Naassila, M., Pitel, A. L., & Vorspan, F. (2022). Alcohol Withdrawal Is an Oxidative Stress Challenge for the Brain: Does It Pave the Way toward Severe Alcohol-Related Cognitive Impairment? *Antioxidants* *11*(10), 2078. https://doi.org/10.3390/antiox11102078

Colombo, E., & Farina, C. (2016). Astrocytes: key regulators of neuroinflammation. *Trends in immunology*, 37(9), 608-620. http://doi.org/10.1016/j.it.2016.06.006

Copeland, A. L., Peltier, M. R., & Geiselman, P. J. (2017). Severity of Menopausal Symptoms and Nicotine Dependence amongst Postmenopausal Women Smokers. *Journal of Smoking Cessation*, *12*(3), 123–130. https://doi.org/10.1017/JSC.2016.14

Corbit, L. H., Chieng, B. C., & Balleine, B. W. (2014). Effects of repeated cocaine exposure on habit learning and reversal by N-

acetylcysteine. *Neuropsychopharmacology*, *39*(8), 1893–1901. https://doi.org/10.1038/npp.2014.37

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., Peana, A. T., Vinci, S., & Acquas, E. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neuroscience and Biobehavioral Reviews*, *36*(1), 404–430. https://doi.org/10.1016/J.NEUBIOREV.2011.07.009

Crews, F. T., Qin, L., Sheedy, D., Vetreno, R. P., & Zou, J. (2013). High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. *Biological Psychiatry*, *73*(7), 602–612. https://doi.org/10.1016/J.BIOPSYCH.2012.09.030

Crews, F. T., & Vetreno, R. P. (2016). Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology*. *233*(9), 1543–1557. https://doi.org/10.1007/s00213-015-3906-1

Crupi, R., Impellizzeri, D., & Cuzzocrea, S. (2019). Role of Metabotropic Glutamate Receptors in Neurological Disorders. *Frontiers in Molecular Neuroscience*, *12*, 20. https://doi.org/10.3389/FNMOL.2019.00020

Cuitavi, J., Lorente, J. D., Campos-Jurado, Y., Polache, A., & Hipólito, L. (2021). Neuroimmune and Mu-Opioid Receptor Alterations in the Mesocorticolimbic System in a Sex-Dependent Inflammatory Pain-Induced Alcohol Relapse-Like Rat Model. *Frontiers in Immunology*, *12*, 1. https://doi.org/10.3389/FIMMU.2021.689453/FULL

Danbolt, N. C. (2001). Glutamate uptake. *Progress in Neurobiology* *65*(1), 1-105. http://doi.org/10.1016/s0301-0082(00)00067-8

Danbolt, N. C., Furness, D. N., & Zhou, Y. (2016). Neuronal vs glial glutamate uptake: resolving the conundrum. *Neurochemistry international*, 98, 29-45. http://doi.org/10.1016/j.neuint.2016.05.009

Das, S. C., Althobaiti, Y. S., Hammad, A. M., Alasmari, F., & Sari, Y. (2022). Role of suppressing GLT-1 and xCT in ceftriaxone-induced attenuation of relapse-like alcohol drinking in alcohol-preferring

rats. *Addiction Biology*, *27*(4), e13178. https://doi.org/10.1111/adb.13178

Das, S. C., Yamamoto, B. K., Hristov, A. M., & Sari, Y. (2015). Ceftriaxone attenuates ethanol drinking and restores extracellular glutamate concentration through normalization of GLT-1 in nucleus accumbens of male alcohol-preferring rats HHS Public Access. *Neuropharmacology*, *97*, 67–74. https://doi.org/10.1016/j.neuropharm.2015.05.009

de Bundel, D., Schallier, A., Loyens, E., Fernando, R., Miyashita, H., van Liefferinge, J., Vermoesen, K., Bannai, S., Sato, H., Michotte, Y., Smolders, I., & Massie, A. (2011). Neurobiology of Disease Loss of System x c Does Not Induce Oxidative Stress But Decreases Extracellular Glutamate in Hippocampus and Influences Spatial Working Memory and Limbic Seizure Susceptibility. *Journal of Neuroscience,* *31*(15), 5792-5803. https://doi.org/10.1523/JNEUROSCI.5465-10.2011

Dean, O., Giorlando, F., & Berk, M. (2011). N-acetylcysteine in psychiatry: Current therapeutic evidence and potential mechanisms of action. *Journal of Psychiatry and Neuroscience,* *36*(2), 78–86. https://doi.org/10.1503/jpn.100057

Deehan, G. A., Hauser, S. R., Wilden, J. A., Truitt, W. A., & Rodd, Z. A. (2013). Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol. *Frontiers in Behavioral Neuroscience*, *7*, 104. https://doi.org/10.3389/FNBEH.2013.00104

Deepmala, Slattery, J., Kumar, N., Delhey, L., Berk, M., Dean, O., Spielholz, C., & Frye, R. (2015). Clinical trials of N-acetylcysteine in psychiatry and neurology: A systematic review. *Neuroscience and Biobehavioral Reviews*, *55*, 294–321. https://doi.org/10.1016/J.NEUBIOREV.2015.04.015

DeSilva, T. M., Kabakov, A. Y., Goldhoff, P. E., Volpe, J. J., & Rosenberg, P. A. (2009). Regulation of glutamate transport in developing rat oligodendrocytes. *Journal of Neuroscience*, 29(24), 7898-7908. http://doi.org/10.1523/JNEUROSCI.6129-08.2009

Ding, Z. M., Rodd, Z. A., Engleman, E. A., Bailey, J. A., Lahiri, D. K., & McBride, W. J. (2013). Alcohol drinking and deprivation alter basal extracellular glutamate concentrations and clearance in the mesolimbic system of alcohol preferring (P) rats. *Addiction Biology*, *18*(2), 297. https://doi.org/10.1111/ADB.12018

Ding, Z. M., Rodd, Z. A., Engleman, E. A., & McBride, W. J. (2009). Sensitization of ventral tegmental area dopamine neurons to the stimulating effects of ethanol. *Alcoholism, Clinical and Experimental Research*, *33*(9), 1571–1581. https://doi.org/10.1111/J.1530-0277.2009.00985.X

Dringen, R. (2000). Metabolism and functions of glutathione in brain. *Progress in Neurobiology*, *62*(6), 649–671. https://doi.org/10.1016/S0301-0082(99)00060-X

Dringen, R., Kussmaul, L., Gutterer, J. M., Hirrlinger, J., & Hamprecht, B. (1999). The glutathione system of peroxide detoxification is less efficient in neurons than in astroglial cells. *Journal of Neurochemistry*, *72*(6), 2523–2530. https://doi.org/10.1046/J.1471-4159.1999.0722523.X

Duailibi, M. S., Cordeiro, Q., Brietzke, E., Ribeiro, M., LaRowe, S., Berk, M., & Trevizol, A. P. (2017). N-acetylcysteine in the treatment of craving in substance use disorders: Systematic review and meta-analysis. *The American Journal on Addictions*, *26*(7), 660–666. https://doi.org/10.1111/AJAD.12620

Ducret, E., Puaud, M., Lacoste, J., Belin-Rauscent, A., Fouyssac, M., Dugast, E., Murray, J. E., Everitt, B. J., Houeto, J. L., & Belin, D. (2016). N-acetylcysteine Facilitates Self-Imposed Abstinence After Escalation of Cocaine Intake. *Biological Psychiatry*, *80*(3), 226–234. https://doi.org/10.1016/j.biopsych.2015.09.019

Echeverry-Alzate, V., Jeanblanc, J., Sauton, P., Bloch, V., Labat, L., Soichot, M., Vorspan, F., & Naassila, M. (2021). Is R(+)-Baclofen the best option for the future of Baclofen in alcohol dependence pharmacotherapy? Insights from the preclinical side. *Addiction Biology*, *26*(2), e12892. https://doi.org/10.1111/ADB.12892

Eidson, L. N., Inoue, K., Young, L. J., Tansey, M. G., & Murphy, A. Z. (2017). Toll-like Receptor 4 Mediates Morphine-Induced Neuroinflammation and Tolerance via Soluble Tumor Necrosis Factor Signaling. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *42*(3), 661–670. https://doi.org/10.1038/NPP.2016.131

Elibol-Can, B., Jakubowska-Dogru, E., Severcan, M., & Severcan, F. (2011). The effects of short-term chronic ethanol intoxication and ethanol withdrawal on the molecular composition of the rat hippocampus by FT-IR spectroscopy. *Alcoholism: Clinical and Experimental Research*, *35*(11), 2050–2062. https://doi.org/10.1111/j.1530-0277.2011.01556.x

Erickson, E. K., Grantham, E. K., Warden, A. S., & Harris, R. A. (2019). Neuroimmune signaling in alcohol use disorder. *Pharmacology Biochemistry and Behavior*, *177*, 34–60. https://doi.org/10.1016/j.pbb.2018.12.007

European Medicines Agency. (2010). Guideline on the development of medicinal products for the treatment of alcohol dependence. *EMEA/CHMP/EWP/20097/2008*.

Ezquer, F., Quintanilla, M. E., Morales, P., Santapau, D., Ezquer, M., Kogan, M. J., Salas-Huenuleo, E., Herrera-Marschitz, M., & Israel, Y. (2019). Intranasal delivery of mesenchymal stem cell-derived exosomes reduces oxidative stress and markedly inhibits ethanol consumption and post-deprivation relapse drinking. *Addiction Biology*, *24*(5), 994–1007. https://doi.org/10.1111/adb.12675

Ezquer, F., Quintanilla, M. E., Morales, P., Santapau, D., Munita, J. M., Moya-Flores, F., Ezquer, M., Herrera-Marschitz, M., & Israel, Y. (2022). A dual treatment blocks alcohol binge-drinking relapse: Microbiota as a new player. *Drug and Alcohol Dependence*, *236*, 109466. https://doi.org/10.1016/J.DRUGALCDEP.2022.109466

Farokhnia, M., Browning, B. D., & Leggio, L. (2019). Prospects for pharmacotherapies to treat alcohol use disorder: An update on recent human studies. *Current Opinion in Psychiatry,* 32(4), 255–265. https://doi.org/10.1097/YCO.0000000000000519

Felipe, J. M., Palombo, P., Bianchi, P. C., Zaniboni, C. R., Anésio, A., Yokoyama, T. S., Engi, S. A., Carneiro-de-Oliveira, P. E., Planeta, C. da S., Leão, R. M., & Cruz, F. C. (2021). Dorsal hippocampus plays a causal role in context-induced reinstatement of alcohol-seeking in rats. *Behavioural Brain Research*, *398*. https://doi.org/10.1016/J.BBR.2020.112978

Fernandes, L. M. P., de Andrade, E. F., Monteiro, M. C., Cartágenes, S. C., Lima, R. R., Prediger, R. D., & Maia, C. S. F. (2017). Ethanol: Neurotoxicity and Brain Disorders. *Addictive Substances and Neurological Disease: Alcohol, Tobacco, Caffeine, and Drugs of Abuse in Everyday Lifestyles*, (pp. 201–215). Academic Press. https://doi.org/10.1016/B978-0-12-805373-7.00020-7

Fernandez-Lizarbe, S., Pascual, M., & Guerri, C. (2009). Activation of Microglia Induced by Ethanol Critical Role of TLR4 Response in the. *The Journal of Immunology*, *183*(7), 4733–4744. https://doi.org/10.4049/jimmunol.0803590

Flores-Bonilla, A., & Richardson, H. N. (2020). Sex Differences in the Neurobiology of Alcohol Use Disorder. *Alcohol Research: Current Reviews*, *40*. https://doi.org/10.35946/arcr.v40.2.04

Foddai, M., Dosia, G., Spiga, S., & Diana, M. (2004). Acetaldehyde increases dopaminergic neuronal activity in the VTA. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *29*(3), 530–536. https://doi.org/10.1038/SJ.NPP.1300326

Foo, J. C., Meinhardt, M. W., Skorodumov, I., & Spanagel, R. (2022). Alcohol solution strength preference predicts compulsive-like drinking behavior in rats. *Alcoholism: Clinical and Experimental Research*, *46*(9), 1710–1719. https://doi.org/10.1111/acer.14910

Franck, J., & Jayaram-Lindström, N. (2013). Pharmacotherapy for alcohol dependence: Status of current treatments. In *Current Opinion in Neurobiology* (Vol. 23, Issue 4, pp. 692–699). https://doi.org/10.1016/j.conb.2013.05.005

Fredriksson, I., Jayaram-Lindström, N., Kalivas, P. W., Melas, P. A., & Steensland, P. (2023). N-acetylcysteine improves impulse control and attenuates relapse-like alcohol intake in long-term drinking rats. *Behavioural Brain Research*, *436*. https://doi.org/10.1016/J.BBR.2022.114089

Fredriksson, I., Jayaram-Lindström, N., Wirf, M., Nylander, E., Nyström, E., Jardemark, K., & Steensland, P. (2015). Evaluation of guanfacine as a potential medication for alcohol use disorder in long-term drinking rats: behavioral and electrophysiological findings. *Neuropsychopharmacology*, 40(5), 1130-1140. http://doi.org/10.1038/npp.2014.294

Fredriksson, I., Venniro, M., Reiner, D. J., Chow, J. J., Bossert, J. M., & Shaham, Y. (2021). Animal Models of Drug Relapse and Craving after Voluntary Abstinence: A Review. *Pharmacological Reviews*, *73*(3), 1050–1083. https://doi.org/10.1124/PHARMREV.120.000191

Froeliger, B., McConnell, P. A., Stankeviciute, N., McClure, E. A., Kalivas, P. W., & Gray, K. M. (2015). The effects of N-Acetylcysteine on frontostriatal resting-state functional connectivity, withdrawal symptoms and smoking abstinence: A double-blind, placebo-controlled fMRI pilot study. *Drug and Alcohol Dependence*, *156*, 234–242. https://doi.org/10.1016/J.DRUGALCDEP.2015.09.021

Furness, D. N., Dehnes, Y., Akhtar, A. Q., Rossi, D. J., Hamann, M., Grutle, N. J., ... & Danbolt, N. C. (2008). A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). *Neuroscience*, 157(1), 80-94. http://doi.org/10.1016/j.neuroscience.2008.08.043

Galbicsek, C. (2021, June 22). *Alcohol-Related Crimes*. Alcohol rehab guide. https://www.alcoholrehabguide.org/alcohol/crimes/

Galduróz, J. C., Prior, P. L., & Ramos, A. C. (2015). Alcohol Withdrawal Syndrome: The Importance of Glutamatergic System. *International Archives of Addiction Research and Medicine*, 1, 006e.

Ghasemitarei, M., Yusupov, M., Razzokov, J., Shokri, B., & Bogaerts, A. (2019). Effect of oxidative stress on cystine transportation by xC‾ antiporter. *Archives of Biochemistry and Biophysics*, *674,* 108114. https://doi.org/10.1016/J.ABB.2019.108114

Gipson, C. D. (2016). Treating Addiction: Unraveling the Relationship Between N-acetylcysteine, Glial Glutamate Transport, and Behavior. *Biological Psychiatry*, *80*(3), e11–e12. https://doi.org/10.1016/J.BIOPSYCH.2016.05.007

Gipson, C. D., Rawls, S., Scofield, M. D., Siemsen, B. M., Bondy, E. O., & Maher, E. E. (2021). Interactions of neuroimmune signaling and glutamate plasticity in addiction. *Journal of Neuroinflammation, 18*(1), 1-23. https://doi.org/10.1186/s12974-021-02072-8

Girard, M., Malauzat, D., & Nubukpo, P. (2019). Serum inflammatory molecules and markers of neuronal damage in alcohol-dependent subjects after withdrawal. *The World Journal of Biological Psychiatry : The Official Journal of the World Federation of Societies of Biological Psychiatry*, *20*(1), 76–90. https://doi.org/10.1080/15622975.2017.1349338

Goenaga, J., Powell, G. L., Leyrer-Jackson, J. M., Piña, J., Phan, S., Prakapenka, A. v., Koebele, S. v., Namba, M. D., McClure, E. A., Bimonte-Nelson, H. A., & Gipson, C. D. (2020). N-acetylcysteine yields sex-specific efficacy for cue-induced reinstatement of nicotine seeking. *Addiction Biology*, *25*(1). https://doi.org/10.1111/adb.12711

Goltseker, K., Hopf, F. W., & Barak, S. (2019). Advances in behavioral animal models of alcohol use disorder. *Alcohol, 74*, 73–82. https://doi.org/10.1016/j.alcohol.2018.05.014

Gonzaga, N. A., Mecawi, A. S., Antunes-Rodrigues, J., de Martinis, B. S., Padovan, C. M., & Tirapelli, C. R. (2015). Ethanol withdrawal increases oxidative stress and reduces nitric oxide bioavailability in the vasculature of rats. *Alcohol*, *49*(1), 47–56. https://doi.org/10.1016/j.alcohol.2014.12.001

Grant, B. F., Goldstein, R. B., Saha, T. D., Patricia Chou, S., Jung, J., Zhang, H., Pickering, R. P., June Ruan, W., Smith, S. M., Huang, B.,

& Hasin, D. S. (2015). Epidemiology of DSM-5 alcohol use disorder results from the national epidemiologic survey on alcohol and related conditions III. *JAMA Psychiatry*, *72*(8), 757–766. https://doi.org/10.1001/jamapsychiatry.2015.0584

Griffin, W. C., Ramachandra, V. S., Knackstedt, L. A., & Becker, H. C. (2015). Repeated cycles of chronic intermittent ethanol exposure increases basal glutamate in the nucleus accumbens of mice without affecting glutamate transport. *Frontiers in Pharmacology*, *6*(FEB). https://doi.org/10.3389/FPHAR.2015.00027

Hagino, Y., Kariura, Y., Manago, Y., Amano, T., Wang, B., Sekiguchi, M., Nishikawa, K., Aoki, S., Wada, K., & Noda, M. (2004). Heterogeneity and potentiation of AMPA type of glutamate receptors in rat cultured microglia. *Glia*, *47*(1), 68–77. https://doi.org/10.1002/GLIA.20034

Hakami, A. Y., Hammad, A. M., & Sari, Y. (2016). Effects of amoxicillin and augmentin on cystine-glutamate exchanger and glutamate transporter 1 isoforms as well as ethanol intake in alcohol-preferring rats. *Frontiers in Neuroscience*, *10*, 171. https://doi.org/10.3389/fnins.2016.00171

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry*, *97*(6), 1634–1658. https://doi.org/10.1111/J.1471-4159.2006.03907.X

Hammad, A. M., Alasmari, F., & Sari, Y. (2021). Effect of Modulation of the Astrocytic Glutamate Transporters' Expression on Cocaine-Induced Reinstatement in Male P Rats Exposed to Ethanol. *Alcohol and Alcoholism*, *56*(2), 210–219. https://doi.org/10.1093/alcalc/agaa104

Han, B., Jones, C. M., Einstein, E. B., Powell, P. A., & Compton, W. M. (2021). Use of Medications for Alcohol Use Disorder in the US: Results From the 2019 National Survey on Drug Use and Health. *JAMA Psychiatry*, *78*(8), 922–924. https://doi.org/10.1001/JAMAPSYCHIATRY.2021.1271

Harper, K. M., Knapp, D. J., Butler, R. K., Cook, C. A., Criswell, H. E., Stuber, G. D., & Breese, G. R. (2019). Amygdala Arginine

Vasopressin Modulates Chronic Ethanol Withdrawal Anxiety-Like Behavior in the Social Interaction Task. *Alcoholism: Clinical and Experimental Research*, *43*(10), 2134–2143. https://doi.org/10.1111/ACER.14163

Harry, G. J., & Kraft, A. D. (2008). Neuroinflammation and Microglia: Considerations and approaches for neurotoxicity assessment. *Expert opinion on drug metabolism & toxicology, 4*(10), 1265-1277. https://doi.org/10.1517/17425255.4.10.1265

Hauser, P., Fuller, B., Ho, S. B., Thuras, P., Kern, S., & Dieperink, E. (2017). The safety and efficacy of baclofen to reduce alcohol use in veterans with chronic hepatitis C: a randomized controlled trial. *Addiction (Abingdon, England)*, *112*(7), 1173–1183. https://doi.org/10.1111/ADD.13787

He, J., & Crews, F. T. (2009). Increased MCP-1 and Microglia in Various Regions of the Human Alcoholic Brain. *Experimental neurology, 210*(2), 349-358. https://doi.org/10.1016/j.expneurol.2007.11.017

Healey, K. L., Kibble, S., Bell, A., Hodges, S., & Swartzwelder, H. S. (2021). Effects of adolescent intermittent ethanol on hippocampal expression of glutamate homeostasis and astrocyte-neuronal tethering proteins in male and female rats. *Journal of Neuroscience Research*, *99*(8), 1908–1921. https://doi.org/10.1002/jnr.24758

Henricks, A. M., Berger, A. L., Lugo, J. M., Baxter-Potter, L. N., Bieniasz, K. v., Craft, R. M., & McLaughlin, R. J. (2016). Sex differences in alcohol consumption and alterations in nucleus accumbens endocannabinoid mRNA in alcohol-dependent rats. *Neuroscience*, *335*, 195–206. https://doi.org/10.1016/J.NEUROSCIENCE.2016.08.032

Hernández, J. A., López-Sánchez, R. C., & Rendón-Ramírez, A. (2016). Lipids and Oxidative Stress Associated with Ethanol-Induced Neurological Damage. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/1543809

Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Granero, L., & Polache, A. (2012). Revisiting the controversial role of salsolinol in the neurobiological effects of ethanol: Old and new vistas. *Neuroscience & Biobehavioral Reviews*, *36*(1), 362–378. https://doi.org/10.1016/J.NEUBIOREV.2011.07.007

Hipolito, L., Sanchez, M., Polache, A., & Granero, L. (2007). Brain Metabolism of Ethanol and Alcoholism: An Update. *Current Drug Metabolism*, *8*(7), 716–727. https://doi.org/10.2174/138920007782109797

Hodebourg, R., Murray, J. E., Fouyssac, M., Puaud, M., Everitt, B. J., & Belin, D. (2019). Heroin seeking becomes dependent on dorsal striatal dopaminergic mechanisms and can be decreased by N-acetylcysteine. *European Journal of Neuroscience*, *50*(3), 2036–2044. https://doi.org/10.1111/ejn.13894

Hölter, S. M., & Spanagel, R. (1999). Effects of opiate antagonist treatment on the alcohol deprivation effect in long-term ethanol-experienced rats. *Psychopharmacology*, *145*(4), 360–369. https://doi.org/10.1007/S002130051069

Huang, M. C., Chen, C. H., Peng, F. C., Tang, S. H., & Chen, C. C. (2009). Alterations in Oxidative Stress Status During Early Alcohol Withdrawal in Alcoholic Patients. *Journal of the Formosan Medical Association*, *108*(7), 560–569. https://doi.org/10.1016/S0929-6646(09)60374-0

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchanan, M. M., Zhao, T. X., Slivka, P. F., Coats, B. D., Rezvani, N., Wieseler, J., Hughes, T. S., Landgraf, K. E., Chan, S., Fong, S., Phipps, S., Falke, J. J., Leinwand, L. A., Maier, S. F., Yin, H., … Watkins, L. R. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain, Behavior, and Immunity*, *24*(1), 83–95. https://doi.org/10.1016/J.BBI.2009.08.004

Israel, Y., Quintanilla, M. E., Ezquer, F., Morales, P., Santapau, D., Berríos-Cárcamo, P., Ezquer, M., Olivares, B., & Herrera-Marschitz, M. (2021). Aspirin and N-acetylcysteine co-administration markedly inhibit chronic ethanol intake and block relapse binge drinking: Role of neuroinflammation-oxidative stress self-

perpetuation. *Addiction Biology*, *26*(1). https://doi.org/10.1111/adb.12853

Jaworski, J., Kalita, K., & Knapska, E. (2018). C-Fos and neuronal plasticity: The aftermath of kaczmarek's theory. *Acta Neurobiologiae Experimentalis, 78*(4), 287–296. https://doi.org/10.21307/ane-2018-027

Johnsen-Soriano, S., Bosch-Morell, F., Miranda, M., Asensio, S., Barcia, J. M., Romá, J., Monfort, P., Felipo, V., & Romero, F. J. (2007). Ebselen Prevents Chronic Alcohol-Induced Rat Hippocampal Stress and Functional Impairment. *Alcoholism: Clinical and Experimental Research*, *31*(3), 486–492. https://doi.org/10.1111/J.1530-0277.2006.00329.X

Jonas, D. E., Amick, H. R., Feltner, C., Bobashev, G., Thomas, K., Wines, R., Kim, M. M., Shanahan, E., Gass, C. E., Rowe, C. J., & Garbutt, J. C. (2014). Pharmacotherapy for adults with alcohol use disorders in outpatient settings: A systematic review and meta-analysis. *JAMA - Journal of the American Medical Association,* 311(18), 1889–1900. https://doi.org/10.1001/jama.2014.3628

Jung, M. (2015). Alcohol withdrawal from the angle of oxidative stress. *Journal of Drug Addiction, Education, and Eradication, 11*(3/4), 307.

Jung, M. E., & Metzger, D. B. (2010). Alcohol withdrawal and brain injuries: Beyond classical mechanisms. *Molecules, 15*(7), 4984–5011). https://doi.org/10.3390/molecules15074984

Jung, M. E., & Metzger, D. B. (2016). A sex difference in oxidative stress and behavioral suppression induced by ethanol withdrawal in rats. *Behavioural Brain Research*, *314*, 199–214. https://doi.org/10.1016/j.bbr.2016.07.054

Jung, M. E., Rewal, M., Perez, E., Wen, Y., & Simpkins, J. W. (2004). Estrogen protects against brain lipid peroxidation in ethanol-withdrawn rats. *Pharmacology, Biochemistry, and Behavior*, *79*(3), 573–586. https://doi.org/10.1016/J.PBB.2004.09.007

Jung, M. E., Yan, L. J., Forster, M. J., & Simpkins, J. W. (2008). Ethanol withdrawal provokes mitochondrial injury in an estrogen preventable manner. *Journal of Bioenergetics and Biomembranes*, *40*(1), 35–44. https://doi.org/10.1007/s10863-008-9129-y

Kalivas, P. W. (2009). The glutamate homeostasis hypothesis of addiction. *Nature Reviews Neuroscience*, *10*(8), 561–572. https://doi.org/10.1038/nrn2515

Kalivas, P. W., & Volkow, N. D. (2005). The Neural Basis of Addiction: A Pathology of Motivation and Choice. *American Journal of Psychiatry*, *162*(8), 1403-1413. http://doi.org/10.1176/appi.ajp.162.8.1403

Katner, S. N., Magalong, J. G., & Weiss, F. (1999). Reinstatement of alcohol-seeking behavior by drug-associated discriminative stimuli after prolonged extinction in the rat. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *20*(5), 471–479. https://doi.org/10.1016/S0893-133X(98)00084-0

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 inflammasome: an overview of mechanisms of activation and regulation. *International journal of molecular sciences, 20*(13), 3328. https://doi.org/10.3390/ijms20133328

Kera, Y., Kiriyama, T., & Komura, S. (1985). Conjugation of acetaldehyde with cysteinylglycine, the first metabolite in glutathione breakdown by gamma-glutamyltranspeptidase. *Agents and Actions*, *17*(1), 48–52. https://doi.org/10.1007/BF01966681

Knackstedt, L. A., LaRowe, S., Mardikian, P., Malcolm, R., Upadhyaya, H., Hedden, S., Markou, A., & Kalivas, P. W. (2009). The role of cystine-glutamate exchange in nicotine dependence in rats and humans. *Biological Psychiatry*, *65*(10), 841–845. https://doi.org/10.1016/J.BIOPSYCH.2008.10.040

Knackstedt, L. A., Melendez, R. I., & Kalivas, P. W. (2010). Ceftriaxone restores glutamate homeostasis and prevents relapse to cocaine

seeking. *Biological Psychiatry*, *67*(1), 81–84. https://doi.org/10.1016/J.BIOPSYCH.2009.07.018

Knapp, D. J., Harper, K. M., Whitman, B. A., Zimomra, Z., & Breese, G. R. (2016). Stress and withdrawal from chronic ethanol induce selective changes in neuroimmune mRNAs in differing brain sites. *Brain Sciences*, *6*(3). https://doi.org/10.3390/brainsci6030025

Koob, G. F., & Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology*, 35(1), 217-238. https://doi.org/10.1016/S2215-0366(16)00104-8

Kornet, M., Goosen, C., & van Ree, J. M. (1991). Effect of naltrexone on alcohol consumption during chronic alcohol drinking and after a period of imposed abstinence in free-choice drinking rhesus monkeys. *Psychopharmacology*, *104*(3), 367–376. https://doi.org/10.1007/BF02246038

Kovács, K. J. (2008). Measurement of Immediate-Early Gene Activation-c-fos and Beyond. *Journal of Neuroendocrinology*, *20*(6), 665–672. https://doi.org/10.1111/J.1365-2826.2008.01734.X

Kraft, A. D., & Harry, G. J. (2011). Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. *International journal of environmental research and public health*, 8(7), 2980-3018. http://doi.org/10.3390/ijerph8072980

Kranzler, H. R., & Soyka, M. (2018). Diagnosis and pharmacotherapy of alcohol use disorder a review. *JAMA - Journal of the American Medical Association* *320*(8), 815–824. https://doi.org/10.1001/jama.2018.11406

Kuhn, B. N., Kalivas, P. W., & Bobadilla, A. C. (2019). Understanding Addiction Using Animal Models. In *Frontiers in Behavioral Neuroscience* *13*, 262. https://doi.org/10.3389/fnbeh.2019.00262

Kupchik, Y. M., Moussawi, K., Tang, X. C., Wang, X., Kalivas, B. C., Kolokithas, R., Ogburn, K. B., & Kalivas, P. W. (2012). The effect of N-acetylcysteine in the nucleus accumbens on neurotransmission and relapse to cocaine. *Biological Psychiatry*,

71(11), 978–986. https://doi.org/10.1016/j.biopsych.2011.10.024

LaRowe, S. D., Kalivas, P. W., Nicholas, J. S., Randall, P. K., Mardikian, P. N., & Malcolm, R. J. (2013). A double-blind placebo-controlled trial of N-acetylcysteine in the treatment of cocaine dependence. *The American Journal on Addictions*, *22*(5), 443–452. https://doi.org/10.1111/J.1521-0391.2013.12034.X

Laverde, C. F., Morais-Silva, G., Amaral, V. C. S., & Marin, M. T. (2021). Effects of N-acetylcysteine treatment on ethanol's rewarding properties and dopaminergic alterations in mesocorticolimbic and nigrostriatal pathways. *Behavioural Pharmacology*, 239–250. https://doi.org/10.1097/FBP.0000000000000613

Lê, A. D., & Shaham, Y. (2002). Neurobiology of relapse to alcohol in rats. *Pharmacology and Therapeutics*, *94*(1–2), 137–156. https://doi.org/10.1016/S0163-7258(02)00200-0

Lebourgeois, S., González-Marín, M. C., Antol, J., Naassila, M., & Vilpoux, C. (2019). Evaluation of N-acetylcysteine on ethanol self-administration in ethanol-dependent rats. *Neuropharmacology*, *150*, 112–120. https://doi.org/10.1016/j.neuropharm.2019.03.010

Lebourgeois, S., González-Marín, M. C., Jeanblanc, J., Naassila, M., & Vilpoux, C. (2018). Effect of N-acetylcysteine on motivation, seeking and relapse to ethanol self-administration. *Addiction Biology*, *23*(2), 643–652. https://doi.org/10.1111/adb.12521

Leclercq, S., Cani, P. D., Neyrinck, A. M., Stärkel, P., Jamar, F., Mikolajczak, M., Delzenne, N. M., & de Timary, P. (2012). Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. *Brain, Behavior, and Immunity*, *26*(6), 911–918. https://doi.org/10.1016/J.BBI.2012.04.001

Lee, E., Sidoryk-Wegrzynowicz, M., Yin, Z., Webb, A., Son, D. S., & Aschner, M. (2012). Transforming growth factor-α mediates estrogen-induced upregulation of glutamate transporter GLT-1 in

rat primary astrocytes. *Glia*, *60*(7), 1024–1036. https://doi.org/10.1002/GLIA.22329

Lehre, K. P., & Danbolt, N. C. (1998). The Number of Glutamate Transporter Subtype Molecules at Glutamatergic Synapses: Chemical and Stereological Quantification in Young Adult Rat Brain. *Journal of neuroscience, 18(*21), 8751-8757. http://doi.org/ 10.1523/JNEUROSCI.18-21-08751.1998

Lehre, K. P., Levy, L. M., Ottersen, O. P., Storm-Mathisen, J., & Danbolt, N. C. (1995). Differential Expression of Two Glial Glutamate Transporters in the Rat Brain: Quantitative and Immunocytochemical Observations. *The Journal of Neuroscience 15(*3), 1835-1853. http://doi.org/10.1523/JNEUROSCI.15-03-01835.1995

Leong, K. C., Cox, S., King, C., Becker, H., & Reichel, C. M. (2018). Oxytocin and Rodent Models of Addiction. *International Review of Neurobiology*, *140*, 201–247. https://doi.org/10.1016/BS.IRN.2018.07.007

Lewerenz, J., Klein, M., & Methner, A. (2006). Cooperative action of glutamate transporters and cystine/glutamate antiporter system Xc- protects from oxidative glutamate toxicity. *Journal of Neurochemistry*, *98*(3), 916–925. https://doi.org/10.1111/J.1471-4159.2006.03921.X

Littleton, J. (1995). Acamprosate in alcohol dependence: how does it work? *Addiction (Abingdon, England)*, *90*(9), 1179–1188. https://doi.org/10.1046/J.1360-0443.1995.90911793.X

Louveau, A., Harris, T. H., & Kipnis, J. (2015). Revisiting the Mechanisms of CNS Immune Privilege. *Trends in Immunology*, *36*(10), 569–577. https://doi.org/10.1016/J.IT.2015.08.006

Lovinger, D. M., Partridge, J. G., & Tang, K. C. (2003). Plastic control of striatal glutamatergic transmission by ensemble actions of several neurotransmitters and targets for drugs of abuse. *Annals of the New York Academy of Sciences*, *1003*, 226–240. https://doi.org/10.1196/ANNALS.1300.014

Madayag, A., Lobner, D., Kau, K. S., Mantsch, J. R., Abdulhameed, O., Hearing, M., Grier, M. D., & Baker, D. A. (2007). Repeated N-acetylcysteine administration alters plasticity-dependent effects of cocaine. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(51), 13968–13976. https://doi.org/10.1523/JNEUROSCI.2808-07.2007

Manning, M., Smith, C., Mazerolle, P., & Tomison, A. (2013). *The societal costs of alcohol misuse in Australia*, (454), 1-6.

Martin-Fardon, R., & Weiss, F. (2013). Modeling relapse in animals. *Current Topics in Behavioral Neurosciences*, *13*, 403–432. https://doi.org/10.1007/7854_2012_202

Martí-Prats, L., Orrico, A., Polache, A., & Granero, L. (2015b). Dual motor responses elicited by ethanol in the posterior VTA: Consequences of the blockade of μ-opioid receptors. *Journal of Psychopharmacology (Oxford, England)*, *29*(9), 1029–1034. https://doi.org/10.1177/0269881115598337

Martí-Prats, L., Sánchez-Catalán, M. J., Hipólito, L., Orrico, A., Zornoza, T., Polache, A., & Granero, L. (2010). Systemic administration of D-penicillamine prevents the locomotor activation after intra-VTA ethanol administration in rats. *Neuroscience Letters*, *483*(2), 143–147. https://doi.org/10.1016/J.NEULET.2010.07.081

Martí-Prats, L., Sánchez-Catalán, M. J., Orrico, A., Zornoza, T., Polache, A., & Granero, L. (2013). Opposite motor responses elicited by ethanol in the posterior VTA: the role of acetaldehyde and the non-metabolized fraction of ethanol. *Neuropharmacology*, *72*, 204–214. https://doi.org/10.1016/J.NEUROPHARM.2013.04.047

Martí-Prats, L., Zornoza, T., López-Moreno, J. A., Granero, L., & Polache, A. (2015a). Acetaldehyde sequestration by D-penicillamine prevents ethanol relapse-like drinking in rats: evidence from an operant self-administration paradigm. *Psychopharmacology*, *232*(19), 3597–3606. https://doi.org/10.1007/S00213-015-4011-1

Massie, A., Boillée, S., Hewett, S., Knackstedt, L., & Lewerenz, J. (2015). Main path and byways: Non-vesicular glutamate release by system

xc- as an important modifier of glutamatergic neurotransmission. *Journal of Neurochemistry* *135*(6), 1062–1079. https://doi.org/10.1111/jnc.13348

McClure, E. A., Gipson, C. D., Malcolm, R. J., Kalivas, P. W., & Gray, K. M. (2014). Potential role of N-acetylcysteine in the management of substance use disorders. *CNS Drugs*, *28*(2), 95–106. https://doi.org/10.1007/s40263-014-0142-x

McKetin, R., Dean, O. M., Baker, A. L., Carter, G., Turner, A., Kelly, P. J., & Berk, M. (2017). A potential role for N-acetylcysteine in the management of methamphetamine dependence. *Drug and Alcohol Review*, *36*(2), 153–159. https://doi.org/10.1111/DAR.12414

McKinzie, D. L., Nowak, K. L., Yorger, L., McBride, W. J., Murphy, J. M., Lumeng, L., & Li, T. K. (1998). The alcohol deprivation effect in the alcohol-preferring P rat under free-drinking and operant access conditions. *Alcoholism, Clinical and Experimental Research*, *22*(5), 1170–1176. https://doi.org/10.1111/j.1530-0277.1998.tb03718.x

Melendez, R. I., Hicks, M. P., Cagle, S. S., & Kalivas, P. W. (2005). Ethanol exposure decreases glutamate uptake in the nucleus accumbens. *Alcoholism: Clinical and Experimental Research*, *29*(3), 326–333. https://doi.org/10.1097/01.ALC.0000156086.65665.4D

Melis, M., Enrico, P., Peana, A. T., & Diana, M. (2007). Acetaldehyde mediates alcohol activation of the mesolimbic dopamine system. *The European Journal of Neuroscience*, *26*(10), 2824–2833. https://doi.org/10.1111/J.1460-9568.2007.05887.X

Minozzi, S., Saulle, R., & Rösner, S. (2018). Baclofen for alcohol use disorder. *The Cochrane Database of Systematic Reviews*, *11*(11). https://doi.org/10.1002/14651858.CD012557.PUB2

Mocelin, R., Marcon, M., da Rosa Araujo, A. S., Herrmann, A. P., & Piato, A. (2019). Withdrawal effects following repeated ethanol exposure are prevented by N-acetylcysteine in zebrafish. *Progress in Neuro-*

Psychopharmacology & Biological Psychiatry, *93*, 161–170. https://doi.org/10.1016/J.PNPBP.2019.03.014

Mocelin, R., Marcon, M., D'ambros, S., Herrmann, A. P., da Rosa Araujo, A. S., & Piato, A. (2018). Behavioral and Biochemical Effects of N-Acetylcysteine in Zebrafish Acutely Exposed to Ethanol. *Neurochemical Research*, *43*(2), 458–464. https://doi.org/10.1007/s11064-017-2442-2

Monasterio, N., Vergara, E., & Morales, T. (2014). Hormonal influences on neuroimmune responses in the CNS of females. *Frontiers in Integrative Neuroscience*, *7*(JAN). https://doi.org/10.3389/FNINT.2013.00110

Monte, A. S., da Silva, F. E. R., Lima, C. N. de C., Vasconcelos, G. S., Gomes, N. S., Miyajima, F., Vasconcelos, S. M. M., Gama, C. S., Seeman, M. v., de Lucena, D. F., & Macedo, D. S. (2020). Sex influences in the preventive effects of N-acetylcysteine in a two-hit animal model of schizophrenia. *Journal of Psychopharmacology (Oxford, England)*, *34*(1), 125–136. https://doi.org/10.1177/0269881119875979

Morais-Silva, G., Alves, G. C., & Marin, M. T. (2016). N-acetylcysteine treatment blocks the development of ethanol-induced behavioural sensitization and related ΔFosB alterations. *Neuropharmacology*, *110*, 135–142. https://doi.org/10.1016/j.neuropharm.2016.07.009

Moro, F., Giannotti, G., Caffino, L., Marzo, C. M., di Clemente, A., Fumagalli, F., & Cervo, L. (2020). Lasting reduction of nicotine-seeking behavior by chronic N-acetylcysteine during experimental cue-exposure therapy. *Addiction Biology*, *25*(4). https://doi.org/10.1111/adb.12771

Moro, F., Orrù, A., Marzo, C. M., di Clemente, A., & Cervo, L. (2018). mGluR2/3 mediates short-term control of nicotine-seeking by acute systemic N-acetylcysteine. *Addiction Biology*, *23*(1), 28–40. https://doi.org/10.1111/adb.12443

Murphy-Royal, C., Dupuis, J. P., Varela, J. A., Panatier, A., Pinson, B., Baufreton, J., ... & Oliet, S. H. (2015). Surface diffusion of

astrocytic glutamate transporters shapes synaptic transmission. *Nature neuroscience,* 18(2), 219-226. http://doi.org/10.1038/nn.3901

Murray, J. E., Everitt, B. J., & Belin, D. (2012). N-Acetylcysteine reduces early- and late-stage cocaine seeking without affecting cocaine taking in rats. *Addiction Biology*, *17*(2), 437–440. https://doi.org/10.1111/J.1369-1600.2011.00330.X

Murugan, M., Sivakumar, V., Lu, J., Ling, E. A., & Kaur, C. (2011). Expression of N-methyl D-aspartate receptor subunits in amoeboid microglia mediates production of nitric oxide via NF-κB signaling pathway and oligodendrocyte cell death in hypoxic postnatal rats. *Glia*, *59*(4), 521–539. https://doi.org/10.1002/GLIA.21121

Nagasawa, H., Elberling, J., & DeMaster, E. (1980). Structural requirements for the sequestration of metabolically generated acetaldehyde. *Journal of Medicinal Chemistry*, *23*(2), 140–143. https://doi.org/10.1021/JM00176A007

Namba, M. D., Kupchik, Y. M., Spencer, S. M., Garcia-Keller, C., Goenaga, J. G., Powell, G. L., Vicino, I. A., Hogue, I. B., & Gipson, C. D. (2020). Accumbens neuroimmune signaling and dysregulation of astrocytic glutamate transport underlie conditioned nicotine-seeking behavior. *Addiction Biology*, *25*(5). https://doi.org/10.1111/adb.12797

Namba, M. D., Leyrer-Jackson, J. M., Nagy, E. K., Olive, M. F., & Neisewander, J. L. (2021). Neuroimmune Mechanisms as Novel Treatment Targets for Substance Use Disorders and Associated Comorbidities. In *Frontiers in Neuroscience* (Vol. 15). Frontiers Media S.A. https://doi.org/10.3389/fnins.2021.650785

Namba, M. D., Tomek, S. E., Olive, M. F., Beckmann, J. S., & Gipson, C. D. (2018). The winding road to relapse: Forging a new understanding of cue-induced reinstatement models and their associated neural mechanisms. In *Frontiers in Behavioral Neuroscience* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fnbeh.2018.00017

National Institute for Health and Care Excellence (Great Britain). (2011). *Alcohol-use disorders: diagnosis, assessment and management of harmful drinking (high-risk drinking) and alcohol dependence*. www.nice.org.uk/guidance/cg115

National Institute on Alcohol Abuse and Alcoholism. (2021, April). *Understanding Alcohol Use Disorder*. https://www.niaaa.nih.gov/sites/default/files/publications/Alcohol_Use_Disorder_0.pdf

Nicola, S. M., James Surmeier, D., & Malenka, R. C. (2000). Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. *Annual Review of Neuroscience*, *23*, 185–215. https://doi.org/10.1146/ANNUREV.NEURO.23.1.185

Northcutt, A. L., Hutchinson, M. R., Wang, X., Baratta, M. v., Hiranita, T., Cochran, T. A., Pomrenze, M. B., Galer, E. L., Kopajtic, T. A., Li, C. M., Amat, J., Larson, G., Cooper, D. C., Huang, Y., O'Neill, C. E., Yin, H., Zahniser, N. R., Katz, J. L., Rice, K. C., … Watkins, L. R. (2015). DAT isn't all that: cocaine reward and reinforcement require Toll-like receptor 4 signaling. *Molecular Psychiatry*, *20*(12), 1525–1537. https://doi.org/10.1038/mp.2014.177

Oka, S.-I., Kamata, H., Kamata, K., Yagisawa, H., & Hirata, H. (2000*). N-Acetylcysteine suppresses TNF-induced NF-UB activation through inhibition of IUB kinases. FEBS letters, 472(*2-3), 196-202. http://doi.org/10.1016/s0014-5793(00)01464-2

Orrico, A., Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Zornoza, T., Granero, L., & Polache, A. (2013). Efficacy of D-penicillamine, a sequestering acetaldehyde agent, in the prevention of alcohol relapse-like drinking in rats. *Psychopharmacology*, *228*(4), 563–575. https://doi.org/10.1007/S00213-013-3065-1

Orrico, A., Martí-Prats, L., Cano-Cebrián, M. J., Granero, L., Polache, A., & Zornoza, T. (2014). Improved effect of the combination naltrexone/D-penicillamine in the prevention of alcohol relapse-like drinking in rats. *Journal of Psychopharmacology (Oxford, England)*, *28*(1), 76–81. https://doi.org/10.1177/0269881113515063

Pascual, M., Baliño, P., Aragón, C. M. G., & Guerri, C. (2015). Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: Role of TLR4 and TLR2. *Neuropharmacology, 89,* 352-359. http://doi.org/10.1016/j.neuropharm.2014.10.014

Pati, D., Kelly, K., Stennett, B., Frazier, C. J., & Knackstedt, L. A. (2016). Alcohol consumption increases basal extracellular glutamate in the nucleus accumbens core of Sprague–Dawley rats without increasing spontaneous glutamate release. *European Journal of Neuroscience*, *44*(2), 1896–1905. https://doi.org/10.1111/ejn.13284

Paxinos, G., & Watson, C. (2007). *The rat brain in stereotaxic coordinates*. London: Academic Press.

Peana, A. T., Assaretti, A. R., Muggironi, G., Enrico, P., & Diana, M. (2009). Reduction of Ethanol-Derived Acetaldehyde Induced Motivational Properties by l-Cysteine. *Alcoholism: Clinical and Experimental Research*, *33*(1), 43–48. https://doi.org/10.1111/J.1530-0277.2008.00809.X

Peana, A. T., Giugliano, V., Rosas, M., Sabariego, M., & Acquas, E. (2013). Effects of L-cysteine on reinstatement of ethanol-seeking behavior and on reinstatement-elicited extracellular signal-regulated kinase phosphorylation in the rat nucleus accumbens shell. *Alcoholism, Clinical and Experimental Research*, *37*, E329-E337 https://doi.org/10.1111/J.1530-0277.2012.01877.X

Peana, A. T., Muggironi, G., & Bennardini, F. (2014). Change of cystine/glutamate antiporter expression in ethanol-dependent rats. *Frontiers in Neuroscience*, *8*, 311. https://doi.org/10.3389/fnins.2014.00311

Peana, A. T., Muggironi, G., Calvisi, G., Enrico, P., Mereu, M., Nieddu, M., Boatto, G., & Diana, M. (2010). l-Cysteine reduces oral ethanol self-administration and reinstatement of ethanol-drinking behavior in rats. *Pharmacology, Biochemistry, and Behavior*, *94*(3), 431–437. https://doi.org/10.1016/J.PBB.2009.10.005

Peana, A. T., Muggironi, G., Fois, G. R., Zinellu, M., Sirca, D., & Diana, M. (2012). Effect of (L)-cysteine on acetaldehyde self-administration. *Alcohol (Fayetteville, N.Y.)*, *46*(5), 489–497. https://doi.org/10.1016/J.ALCOHOL.2011.10.004

Peana, A. T., Rosas, M., Porru, S., & Acquas, E. (2016). From Ethanol to Salsolinol: Role of Ethanol Metabolites in the Effects of Ethanol. *Journal of Experimental Neuroscience*, *10*(1), 137–146. https://doi.org/10.4137/JEN.S25099

Pickens, C. L., Airavaara, M., Theberge, F., Fanous, S., Hope, B. T., & Shaham, Y. (2011). Neurobiology of the incubation of drug craving. *Trends in Neurosciences*, *34*(8), 411–420. https://doi.org/10.1016/J.TINS.2011.06.001

Pirino, B. E., Martin, C. R., Carpenter, B. A., Curtis, G. R., Curran-Alfaro, C. M., Samels, S. B., Barker, J. M., Karkhanis, A. N., & Barson, J. R. (2022). Sex-related differences in pattern of ethanol drinking under the intermittent-access model and its impact on exploratory and anxiety-like behavior in Long-Evans rats. *Alcoholism: Clinical and Experimental Research*, *46*(7), 1282–1293. https://doi.org/10.1111/ACER.14853

Plosker, G. L. (2015). Acamprosate: A review of its use in alcohol dependence. *Drugs*, *75*(11), 1255–1268. https://doi.org/10.1007/s40265-015-0423-9

Powell, G. L., Leyrer-Jackson, J. M., Goenaga, J., Namba, M. D., Piña, J., Spencer, S., Stankeviciute, N., Schwartz, D., Allen, N. P., del Franco, A. P., McClure, E. A., Olive, M. F., & Gipson, C. D. (2019). Chronic treatment with N-acetylcysteine decreases extinction responding and reduces cue-induced nicotine-seeking. *Physiological Reports*, *7*(1), e13958. https://doi.org/10.14814/PHY2.13958

Pradhan, G., Melugin, P. R., Wu, F., Fang, H. M., Weber, R., & Kroener, S. (2018). Calcium chloride mimics the effects of acamprosate on cognitive deficits in chronic alcohol-exposed mice. *Psychopharmacology*, *235*(7), 2027–2040. https://doi.org/10.1007/S00213-018-4900-1

Qin, L., He, J., Hanes, R. N., Pluzarev, O., Hong, J.-S., & Crews, F. T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *Journal of neuroinflammation* 5(1), 1-17. https://doi.org/10.1186/1742-2094-5-10

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., Knapp, D. J., & Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*, *55*(5), 453–462. https://doi.org/10.1002/GLIA.20467

Quintanilla, M. E., Ezquer, F., Morales, P., Ezquer, M., Olivares, B., Santapau, D., Herrera-Marschitz, M., & Israel, Y. (2020). N-Acetylcysteine and Acetylsalicylic Acid Inhibit Alcohol Consumption by Different Mechanisms: Combined Protection. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/fnbeh.2020.00122

Quintanilla, M. E., Ezquer, F., Morales, P., Santapau, D., Ezquer, M., Herrera-Marschitz, M., & Israel, Y. (2022). A dual mechanism fully blocks ethanol relapse: Role of vagal innervation. *Addiction Biology*, *27*(2). https://doi.org/10.1111/ADB.13140

Quintanilla, M. E., Israel, Y., Sapag, A., & Tampier, L. (2006). The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. *Addiction Biology*, *11*(3–4), 310–323. https://doi.org/10.1111/J.1369-1600.2006.00030.X

Quintanilla, M. E., Morales, P., Ezquer, F., Ezquer, M., Herrera-Marschitz, M., & Israel, Y. (2018). Commonality of Ethanol and Nicotine Reinforcement and Relapse in Wistar-Derived UChB Rats: Inhibition by N-Acetylcysteine. *Alcoholism: Clinical and Experimental Research*, *42*(10), 1988–1999. https://doi.org/10.1111/acer.13842

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Salinas-Luypaert, C., Herrera-Marschitz, M., & Israel, Y. (2016). Beyond the "First Hit": Marked Inhibition by N-Acetyl Cysteine of Chronic Ethanol Intake But Not of Early Ethanol Intake. Parallel Effects on Ethanol-Induced Saccharin Motivation. *Alcoholism: Clinical and*

Experimental Research, *40*(5), 1044–1051. https://doi.org/10.1111/acer.13031

Ramachandran, V., Watts, L. T., Maffi, S. K., Chen, J., Schenker, S., & Henderson, G. (2003). Ethanol-Induced Oxidative Stress Precedes Mitochondrially Mediated Apoptotic Death of Cultured Fetal Cortical Neurons. *Journal of neuroscience research 74*(4), 577-588. http://doi.org/10.1002/jnr.10767

Ramirez-Niño, A. M., D'Souza, M. S., & Markou, A. (2013). N-acetylcysteine decreased nicotine self-administration and cue-induced reinstatement of nicotine seeking in rats: comparison with the effects of N-acetylcysteine on food responding and food seeking. *Psychopharmacology*, *225*(2), 473–482. https://doi.org/10.1007/S00213-012-2837-3

Rao, P. S. S., Bell, R. L., Engleman, E. A., & Sari, Y. (2015a). Targeting glutamate uptake to treat alcohol use disorders. *Frontiers in Neuroscience 9,* 144. https://doi.org/10.3389/fnins.2015.00144

Rao, P. S. S., Saternos, H., Goodwani, S., & Sari, Y. (2015b). Effects of ceftriaxone on GLT1 isoforms, xCT and associated signaling pathways in P rats exposed to ethanol. *Psychopharmacology*, *232*(13), 2333–2342. https://doi.org/10.1007/s00213-015-3868-3

Reddy, V. D., Padmavathi, P., Kavitha, G., Saradamma, B., & Varadacharyulu, N. (2013). Alcohol-induced oxidative/nitrosative stress alters brain mitochondrial membrane properties. *Molecular and Cellular Biochemistry*, *375*(1–2), 39–47. https://doi.org/10.1007/s11010-012-1526-1

Reilly, M. T., Noronha, A., & Warren, K. (2014). Perspectives on the neuroscience of alcohol from the National Institute on Alcohol Abuse and Alcoholism. *Handbook of Clinical Neurology*, *125*, 15–29. https://doi.org/10.1016/B978-0-444-62619-6.00002-1

Reissner, K. J., Gipson, C. D., Tran, P. K., Knackstedt, L. A., Scofield, M. D., & Kalivas, P. W. (2015). Glutamate transporter GLT-1 mediates

N-acetylcysteine inhibition of cocaine reinstatement. *Addiction Biology*, *20*(2), 316–323. https://doi.org/10.1111/adb.12127

Reissner, K. J., & Kalivas, P. W. (2010). Using glutamate homeostasis as a target for treating addictive disorders. *Behavioural Pharmacology*, *21*(5–6), 514–522. https://doi.org/10.1097/FBP.0B013E32833D41B2

Reynaud, M., Aubin, H. J., Trinquet, F., Zakine, B., Dano, C., Dematteis, M., Trojak, B., Paille, F., & Detilleux, M. (2017). A Randomized, Placebo-Controlled Study of High-Dose Baclofen in Alcohol-Dependent Patients-The ALPADIR Study. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *52*(4), 439–446. https://doi.org/10.1093/ALCALC/AGX030

Roberto, M., Gilpin, N. W., & Siggins, G. R. (2012). The Central Amygdala and Alcohol: Role of g-Aminobutyric Acid, Glutamate, and Neuropeptides. *Cold Spring Harbor Perspectives in Medicine*, *2*(12), a012195. https://doi.org/10.1101/cshperspect.a012195

Roberto, M., Kirson, D., & Khom, S. (2021). The Role of the Central Amygdala in Alcohol Dependence. *Cold Spring Harbor Perspectives in Medicine*, *11*(2), 1–23. https://doi.org/10.1101/CSHPERSPECT.A039339

Roberto, M., Schweitzer, P., Madamba, S. G., Stouffer, D. G., Parsons, L. H., & Siggins, G. R. (2004). Acute and Chronic Ethanol Alter Glutamatergic Transmission in Rat Central Amygdala: An In Vitro and In Vivo Analysis. *Journal of Neuroscience*, *24*(7), 1594–1603. https://doi.org/10.1523/JNEUROSCI.5077-03.2004

Rodd, Z. A., Bell, R. L., Sable, H. J. K., Murphy, J. M., & McBride, W. J. (2004). Recent advances in animal models of alcohol craving and relapse. *Pharmacology, Biochemistry, and Behavior*, *79*(3), 439–450. https://doi.org/10.1016/J.PBB.2004.08.018

Rolland, B., Labreuche, J., Duhamel, A., Deheul, S., Gautier, S., Auffret, M., Pignon, B., Valin, T., Bordet, R., & Cottencin, O. (2015). Baclofen for alcohol dependence: Relationships between baclofen and alcohol dosing and the occurrence of major

sedation. *European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology*, *25*(10), 1631–1636. https://doi.org/10.1016/J.EURONEURO.2015.05.008

Rossetti, Z. L., & Carboni, S. (1995). Ethanol withdrawal is associated with increased extracellular glutamate in the rat striatum. *European Journal of Pharmacology*, *283*(1–3), 177–183. https://doi.org/10.1016/0014-2999(95)00344-K

Russell, W., & Burch, R. (1959). *The principles of humane experimental technique*. Methuen.

Saellstroem Baum, S., Huebner, A., Krimphove, M., Morgenstern, R., Badawy, A. A. B., & Spies, C. D. (2006). Nicotine Stimulation on Extracellular Glutamate Levels in the Nucleus Accumbens of Ethanol-withdrawn Rats In Vivo. *Alcoholism: Clinical and Experimental Research*, 30(8), 1414-1421. http://doi.org/10.1111/j.1530-0277.2006.00169.x

Salaspuro, V., Hietala, J., Kaihovaara, P., Pihlajarinne, L., Marvola, M., & Salaspuro, M. (2002). Removal of acetaldehyde from saliva by a slow-release buccal tablet of L-cysteine. *International Journal of Cancer*, *97*(3), 361–364. https://doi.org/10.1002/IJC.1620

Salimov, R. M., & Salimova, N. B. (1993). The alcohol-deprivation effect in hybrid mice. *Drug and Alcohol Dependence*, *32*(2), 187–191. https://doi.org/10.1016/0376-8716(93)80012-4

Samson, H. H., & Doyle, T. F. (1985). Oral ethanol self-administration in the rat: effect of naloxone. *Pharmacology, Biochemistry, and Behavior*, *22*(1), 91–99. https://doi.org/10.1016/0091-3057(85)90491-5

Sanchez-Alavez, M., Nguyen, W., Mori, S., Wills, D. N., Otero, D., Ehlers, C. L., & Conti, B. (2019). Time course of microglia activation and brain and blood cytokine/chemokine levels following chronic ethanol exposure and protracted withdrawal in rats HHS Public Access. *Alcohol*, *76*, 37–45. https://doi.org/10.1016/j.alcohol.2018.07.005

Sánchez-Catalán, M. J., Hipólito, L., Guerri, C., Granero, L., & Polache, A. (2008). Distribution and differential induction of CYP2E1 by ethanol and acetone in the mesocorticolimbic system of rat. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *43*(4), 401–407. https://doi.org/10.1093/ALCALC/AGN012

Sánchez-Catalán, M. J., Hipólito, L., Zornoza, T., Polache, A., & Granero, L. (2009). Motor stimulant effects of ethanol and acetaldehyde injected into the posterior ventral tegmental area of rats: Role of opioid receptors. *Psychopharmacology*, *204*(4), 641–653. https://doi.org/10.1007/S00213-009-1495-6

Sanchis-Segura, C., & Spanagel, R. (2006). Behavioural assessment of drug reinforcement and addictive features in rodents: An overview. *Addiction Biology*, *11*(1), 2–38. https://doi.org/10.1111/J.1369-1600.2006.00012.X

Sari, Y., Sreemantula, S. N., Lee, M. R., & Choi, D. S. (2013). Ceftriaxone treatment affects the levels of GLT1 and ENT1 as well as ethanol intake in alcohol-preferring rats. *Journal of Molecular Neuroscience*, *51*(3), 779–787. https://doi.org/10.1007/s12031-013-0064-y

Sato, H., Tamba, M., Ishii, T., & Bannai, S. (1999). Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *The Journal of Biological Chemistry*, *274*(17), 11455–11458. https://doi.org/10.1074/JBC.274.17.11455

Schneider, R., Bandiera, S., Souza, D. G., Bellaver, B., Caletti, G., Quincozes-Santos, A., Elisabetsky, E., & Gomez, R. (2017). N-acetylcysteine Prevents Alcohol Related Neuroinflammation in Rats. *Neurochemical Research*, *42*(8), 2135–2141. https://doi.org/10.1007/s11064-017-2218-8

Schneider, R., Santos, C. F., Clarimundo, V., Dalmaz, C., Elisabetsky, E., & Gomez, R. (2015). N-acetylcysteine prevents behavioral and biochemical changes induced by alcohol cessation in rats. *Alcohol (Fayetteville, N.Y.)*, *49*(3), 259–263. https://doi.org/10.1016/J.ALCOHOL.2015.01.009

Scofield, M. D., & Kalivas, P. W. (2014). Astrocytic dysfunction and addiction: consequences of impaired glutamate homeostasis. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *20*(6), 610–622. https://doi.org/10.1177/1073858413520347

Scolaro, B., Delwing-De Lima, D., da Cruz, J. G. P., & Delwing-Dal Magro, D. (2012). Mate tea prevents oxidative stress in the blood and hippocampus of rats with acute or chronic ethanol administration. *Oxidative Medicine and Cellular Longevity*, *2012*. https://doi.org/10.1155/2012/314758

Seo, D., & Sinha, R. (2014). The neurobiology of alcohol craving and relapse. *Handbook of clinical neurology*, 125, 355-368. https://doi.org/10.1016/B978-0-444-62619-6.00021-5

Shaham, Y., Shalev, U., Lu, L., de Wit, H., & Stewart, J. (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology*, *168*(1–2), 3–20. https://doi.org/10.1007/S00213-002-1224-X

Siemsen, B. M., Landin, J. D., McFaddin, J. A., Hooker, K. N., Chandler, L. J., Scofield, M. D., & Benjamin Siemsen, C. M. (2021). Chronic intermittent ethanol and lipopolysaccharide exposure differentially alter Iba1-derived microglia morphology in the prelimbic cortex and nucleus accumbens core of male Long-Evans rats HHS Public Access. *Journal of Neuroscience Research*, *99*(8), 1922–1939. https://doi.org/10.1002/jnr.24683

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., & Bartlett, S. E. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcoholism, Clinical and Experimental Research*, *32*(10), 1816–1823. https://doi.org/10.1111/J.1530-0277.2008.00753.X

Sinclair, J. D., & Senter, R. J. (1968). Development of an alcohol-deprivation effect in rats. *Quarterly Journal of Studies on Alcohol*, *29*(4), 863–867. https://doi.org/10.15288/qjsa.1968.29.863

Sinclair, J. D., & Tiihonen, K. (1988). Lack of alcohol-deprivation effect in AA rats. *Alcohol (Fayetteville, N.Y.)*, *5*(1), 85–87. https://doi.org/10.1016/0741-8329(88)90048-1

Sinclair, J. D., Walker, S., & Jordan, W. (1973). Behavioral and Physiological Changes Associated with Various Durations of Alcohol Deprivation in Rats. *Quarterly journal of studies in alcohol, 34*(3), 744–757. https://doi.org/10.15288/QJSA.1973.34.744

Sirca, D., Enrico, P., Mereu, M., Peana, A. T., & Diana, M. (2011). l-cysteine Prevents Ethanol-Induced Stimulation of Mesolimbic Dopamine Transmission. *Alcoholism: Clinical and Experimental Research*, *35*(5), 862–869. https://doi.org/10.1111/J.1530-0277.2010.01416.X

Smaga, I., Frankowska, M., & Filip, M. (2021). N-acetylcysteine in substance use disorder: a lesson from preclinical and clinical research. *Pharmacological Reports* *73*(5), 1205–1219. https://doi.org/10.1007/s43440-021-00283-7

Sohi, I., Franklin, A., Chrystoja, B., Wettlaufer, A., Rehm, J., & Shield, K. (2021). The Global Impact of Alcohol Consumption on Premature Mortality and Health in 2016. *Nutrients*, *13*(9). https://doi.org/10.3390/NU13093145

Soldelberg, T. (2022, July 20). *15.7: Redox Reactions of Thiols and Disulfides*. LibreTexts Chemistry. https://chem.libretexts.org/@go/page/106651

Sommavilla, M., Sánchez-Villarejo, M. V., Almansa, I., Sánchez-Vallejo, V., Barcia, J. M., Romero, F. J., & Miranda, M. (2012). The effects of acute ethanol exposure and ageing on rat brain glutathione metabolism. *Free radical research, 46*(9), 1076–1081. https://doi.org/10.3109/10715762.2012.688963

Spanagel, R. (2017). Animal models of addiction. *Dialogues in clinical neuroscience.* http://doi.org/10.31887/DCNS.2017.19.3/rspanagel

Spanagel, R., & Hölter, S. M. (1999). Long-term alcohol self-administration with repeated alcohol deprivation phases: An

animal model of alcoholism? *Alcohol and Alcoholism*, *34*(2), 231–243. https://doi.org/10.1093/ALCALC/34.2.231

Spanagel, R., & Kiefer, F. (2008). Drugs for relapse prevention of alcoholism: ten years of progress. *Trends in Pharmacological Sciences*, *29*(3), 109–115. https://doi.org/10.1016/J.TIPS.2007.12.005

Spanagel, R., & Vengeliene, V. (2013). New pharmacological treatment strategies for relapse prevention. *Current Topics in Behavioral Neurosciences*, *13*, 583–609. https://doi.org/10.1007/7854_2012_205

Spanagel, R., Vengeliene, V., Jandeleit, B., Fischer, W. N., Grindstaff, K., Zhang, X., Gallop, M. A., Krstew, E. v., Lawrence, A. J., & Kiefer, F. (2014). Acamprosate produces its anti-relapse effects via calcium. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *39*(4), 783–791. https://doi.org/10.1038/NPP.2013.264

Squeglia, L. M., Tomko, R. L., Baker, N. L., McClure, E. A., Book, G. A., & Gray, K. M. (2018). The effect of N-acetylcysteine on alcohol use during a cannabis cessation trial. *Drug and Alcohol Dependence*, *185*, 17–22. https://doi.org/10.1016/j.drugalcdep.2017.12.005

Stennett, B. A., Frankowski, J. C., Peris, J., & Knackstedt, L. A. (2017). Ceftriaxone reduces alcohol intake in outbred rats while upregulating xCT in the nucleus accumbens core. *Pharmacology, Biochemistry, and Behavior*, *159*, 18–23. https://doi.org/10.1016/J.PBB.2017.07.001

Stewart, R. B., & Li, T. K. (1997). The Neurobiology of Alcoholism in Genetically Selected Rat Models. *Alcohol Health and Research World*, *21*(2), 169.

Surmeier, D. J., Ding, J., Day, M., Wang, Z., & Shen, W. (2007). D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends in Neurosciences*, *30*(5), 228–235. https://doi.org/10.1016/J.TINS.2007.03.008

Tapia-Rojas, C., Pérez, M. J., Jara, C., Vergara, E. H., & Quintanilla, R. A. (2018). Ethanol Consumption Affects Neuronal Function: Role of the Mitochondria. In E. Taskin, C. Guven, & Y. Sevgiler (Eds.), *Mitochondrial Diseases*. InTech. https://doi.org/10.5772/intechopen.71611

Tiwari, V., & Chopra, K. (2013). Resveratrol abrogates alcohol-induced cognitive deficits by attenuating oxidative–nitrosative stress and inflammatory cascade in the adult rat brain. *Neurochemistry International*, *62*(6), 861–869. https://doi.org/10.1016/J.NEUINT.2013.02.012

Tiwari, V., Kuhad, A., & Chopra, K. (2009). Suppression of neuro-inflammatory signaling cascade by tocotrienol can prevent chronic alcohol-induced cognitive dysfunction in rats. *Behavioural Brain Research*, *203*(2), 296–303. https://doi.org/10.1016/J.BBR.2009.05.016

Trotti, D., Danbolt, N. C., & Volterra, A. (1988). Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *Trends in pharmacological sciences* *19*(8), 328-334. http://doi.org/ 10.1016/s0165-6147(98)01230-9

Tsai, G. Y., Cui, J. Z., Syed, H., Xia, Z., Ozerdem, U., Mcneill, J. H., & Matsubara, J. A. (2009). Effect of N-acetylcysteine on the early expression of inflammatory markers in the retina and plasma of diabetic rats. *Clin Exp Ophthalmol*, *37*(2), 223–231. https://doi.org/10.1111/j.1442-9071.2009.02000.x

Uys, J. D., Mulholland, P. J., & Townsend, D. M. (2014). Glutathione and redox signaling in substance abuse. *Biomedicine and Pharmacotherapy* *68*(6), 799–807. https://doi.org/10.1016/j.biopha.2014.06.001

Vallés, S. L., Blanco, A. M., Pascual, M., & Guerri, C. (2004). Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. *Brain Pathology*, *14*(4), 365–371. https://doi.org/10.1111/J.1750-3639.2004.TB00079.X

Vengeliene, V., Bachteler, D., Danysz, W., & Spanagel, R. (2005). The role of the NMDA receptor in alcohol relapse: a pharmacological mapping study using the alcohol deprivation effect. *Neuropharmacology*, *48*(6), 822–829. https://doi.org/10.1016/J.NEUROPHARM.2005.01.002

Vengeliene, V., Bilbao, A., & Spanagel, R. (2014). The alcohol deprivation effect model for studying relapse behavior: A comparison between rats and mice. *Alcohol*, *48*(3), 313–320. https://doi.org/10.1016/j.alcohol.2014.03.002

Vengeliene, V., Heidbreder, C. A., & Spanagel, R. (2007). The effects of lamotrigine on alcohol seeking and relapse. *Neuropharmacology*, *53*(8), 951–957. https://doi.org/10.1016/j.neuropharm.2007.09.006

Vengeliene, V., Siegmund, S., Singer, M. v., Sinclair, J. D., Li, T. K., & Spanagel, R. (2003). A comparative study on alcohol-preferring rat lines: effects of deprivation and stress phases on voluntary alcohol intake. *Alcoholism, Clinical and Experimental Research*, *27*(7), 1048–1054. https://doi.org/10.1097/01.ALC.0000075829.81211.0C

Vetreno, R. P., Qin, L., & Crews, F. T. (2013). Increased Receptor for Advanced Glycation End Product Expression in the Human Alcoholic Prefrontal Cortex is Linked to Adolescent Drinking. *Neurobiology of Disease*. https://doi.org/10.1016/j.nbd.2013.07.002

Vollstädt-Klein, S., Wichert, S., Rabinstein, J., Bühler, M., Klein, O., Ende, G., Hermann, D., & Mann, K. (2010). Initial, habitual and compulsive alcohol use is characterized by a shift of cue processing from ventral to dorsal striatum. *Addiction (Abingdon, England)*, *105*(10), 1741–1749. https://doi.org/10.1111/J.1360-0443.2010.03022.X

Wang, Z., Kai, L., Day, M., Ronesi, J., Yin, H. H., Ding, J., Tkatch, T., Lovinger, D. M., & Surmeier, D. J. (2006). Dopaminergic control of corticostriatal long-term synaptic depression in medium spiny neurons is mediated by cholinergic interneurons. *Neuron*, *50*(3), 443–452. https://doi.org/10.1016/J.NEURON.2006.04.010

Warden, A., Erickson, E., Robinson, G., Harris, R. A., & Mayfield, R. D. (2016). The neuroimmune transcriptome and alcohol dependence: potential for targeted therapies. *Pharmacogenomics*, *17*(18), 2081. https://doi.org/10.2217/PGS-2016-0062

Weiland, A., Garcia, S., & Knackstedt, L. A. (2015). Ceftriaxone and cefazolin attenuate the cue-primed reinstatement of alcohol-seeking. *Frontiers in Pharmacology*, *6*, 44. https://doi.org/10.3389/fphar.2015.00044

Weiss, F., Ciccocioppo, R., Parsons, L. H., Katner, S., Liu, X. I. U., Zorrilla, E. P., Valdez, G. R., Ben-Shahar, O., Angeletti, S., & Richter, R. R. (2001). Compulsive drug-seeking behavior and relapse: Neuroadaptation, stress, and conditioning factors. *Annals of the New York Academy of Sciences*, *937*, 1–26. https://doi.org/10.1111/j.1749-6632.2001.tb03556.x

Whitman, B. A., Knapp, D. J., Werner, D. F., Crews, F. T., & Breese, G. R. (2013). The Cytokine mRNA Increase Induced by Withdrawal from Chronic Ethanol in the Sterile Environment of Brain is Mediated by CRF and HMGB1 Release. *Alcoholism: Clinical and Experimental Research*, *37*(12), 2086–2097. https://doi.org/10.1111/ACER.12189

Witkiewitz, K., Litten, R. Z., & Leggio, L. (2019). Advances in the science and treatment of alcohol use disorder. *Sciences Advances*, *5*(9), eaax4043. http://doi.org/10.1126/sciadv.aax4043

Wolffgramm, J., & Heyne, A. (1995). From controlled drug intake to loss of control: the irreversible development of drug addiction in the rat. *Behavioural Brain Research*, *70*(1), 77–94. https://doi.org/10.1016/0166-4328(95)00131-C

Womersley, J. S., Townsend, D. M., Kalivas, P. W., & Uys, J. D. (2019). Targeting redox regulation to treat substance use disorder using N-acetylcysteine. *European Journal of Neuroscience, 50*(3), 2538–2551. https://doi.org/10.1111/ejn.14130

Womersley, J. S., & Uys, J. D. (2016). S-Glutathionylation and Redox Protein Signaling in Drug Addiction. *Progress in Molecular Biology*

and Translational Science, *137*, 87–121. https://doi.org/10.1016/bs.pmbts.2015.10.001

Woodcock, E. A., Lundahl, L. H., Khatib, D., Stanley, J. A., & Greenwald, M. K. (2021). N-acetylcysteine reduces cocaine-seeking behavior and anterior cingulate glutamate/glutamine levels among cocaine-dependent individuals. *Addiction Biology*, *26*(2). https://doi.org/10.1111/adb.12900

World Health Organization. (2018). *Global status report on alcohol and health 2018.* World Health Organization.

Wu, D., & Cederbaum, A. I. (2003). Alcohol, oxidative stress, and free radical damage. *Alcohol Research and Health, 27*(4), 277–284. https://doi.org/10.1079/pns2006496

Ye, Z. W., Zhang, J., Townsend, D. M., & Tew, K. D. (2015). Oxidative stress, redox regulation and diseases of cellular differentiation. *Biochimica et Biophysica Acta*, *1850*(8), 1607–1621. https://doi.org/10.1016/J.BBAGEN.2014.11.010

Yen, C. H., Ho, P. S., Yeh, Y. W., Liang, C. S., Kuo, S. C., Huang, C. C., Chen, C. Y., Shih, M. C., Ma, K. H., Sung, Y. F., Lu, R. B., & Huang, S. Y. (2017). Differential cytokine levels between early withdrawal and remission states in patients with alcohol dependence. *Psychoneuroendocrinology*, *76*, 183–191. https://doi.org/10.1016/J.PSYNEUEN.2016.10.015

Zhang, W.-H., Cao, K.-X., Ding, Z.-B., Yang, J.-L., Pan, B.-X., & Xue, Y.-X. (2019). Role of prefrontal cortex in the extinction of drug memories. *Psychopharmacoloty, 236*(1), 463-477. https://doi.org/10.1007/s00213-018-5069-3

Zhao, Y. N., Wang, F., Fan, Y. X., Ping, G. F., Yang, J. Y., & Wu, C. F. (2013). Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behavioural Brain Research*, *236*(1), 270–282. https://doi.org/10.1016/j.bbr.2012.08.052

Zhou, W., & Kalivas, P. W. (2008). N-Acetylcysteine Reduces Extinction Responding and Induces Enduring Reductions in Cue- and Heroin-

Induced Drug-Seeking. *Biological Psychiatry*, *63*(3), 338–340. https://doi.org/10.1016/j.biopsych.2007.06.008

Zimatkin, S. M., Pronko, S. P., Vasiliou, V., Gonzalez, F. J., & Deitrich, R. A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcoholism, Clinical and Experimental Research*, *30*(9), 1500–1505. https://doi.org/10.1111/J.1530-0277.2006.00181.X

Zornoza, T., Cano-Cebrián, M. J., Martínez-García, F., Polache, A., & Granero, L. (2005). Hippocampal dopamine receptors modulate cFos expression in the rat nucleus accumbens evoked by chemical stimulation of the ventral hippocampus. *Neuropharmacology*, *49*(7), 1067–1076. https://doi.org/10.1016/J.NEUROPHARM.2005.06.005

Zou, J. Y., & Crews, F. T. (2014). Release of Neuronal HMGB1 by Ethanol through Decreased HDAC Activity Activates Brain Neuroimmune Signaling. *PLOS ONE*, *9*(2), e87915. https://doi.org/10.1371/JOURNAL.PONE.0087915