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PROGRAMA DE DOCTORADO EN BIOMEDICINA

BINGE DRINKING MODELS IN RATS: ASSESSING THE INFLUENCE OF BINGE EATING AND SIGMA-1 RECEPTOR

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Leandro Ruiz Leyva

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UNIVERSIDAD DE GRANADA

Facultad de Medicina

Departamento de Farmacología e Instituto de Neurociencias Federico Olóriz

Directores:

CRUZ MIGUEL CENDÁN MARTÍNEZ

IGNACIO MORÓN HENCHE

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Introduction

1. An ancient companion

In most modern societies, it is safe to say that alcohol plays a key role in our lives, as it is an important aspect of many cultures, religions and interpersonal relationships. Therefore, it is no surprise that alcohol is one of the most widely consumed psychoactive substances. However, our relationship with alcohol is not a new one. It is believed that the consumption of ethanol dates back to well before the earliest stages of human history. In fact, paleogenetic scientific models propose that 10 million years ago, a new mutation facilitated a significant improvement in our ancestors' ability to metabolize alcohol, thus acquiring the adaptative advantage of being able to feed on ripe fruit that naturally contains it (Carrigan et al., 2015).

The first solid evidence of alcoholic beverages preparations was discovered in a tomb in northern China, where a vessel containing an alcoholic brew was found (see Figure 1). This vessels dates back to around 7000 BC (Wang et al., 2021). However, due to the fact that alcoholic fermentation occurs naturally from the sugars in different foodstuff, human consumption of alcohol actually appeared long before that. Since then, myriad archaeological records have been found all over the world. Sumeria, considered the first civilization in history, is where the oldest written records of the role that alcoholic beverages played in society were discovered.

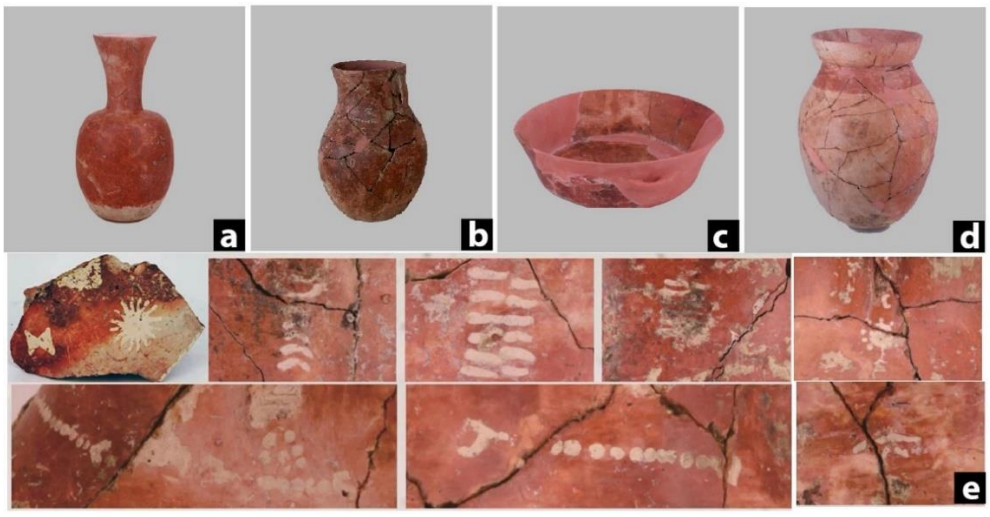

Figure 1. Different types of pottery found in a 9000-year-old burial in Qiaotou (China) that contained beer made of fermented rice (a – d) and details of the patterns painted on them (e) (Source: Wang et al., 2021).

Throughout history, alcohol and humans have had a love-hate relationship. The Sumerians themselves, had deities related to alcoholic drinks (i.e., Ninkasi, “goddess of brewing”), art pieces depicting scenes of alcohol consumption, literature where alcohol is cited (i.e., *Gilgamesh*, 2000 BC) and even legislation associated with it.

There are records from the ancient Egyptian civilization that how advanced their winemaking techniques were. They not only made different types of wines but also labelled them in a way that nowadays we know as *appellation*, which shows the importance of this product in that society.

If one goes forward in time to Greek polis, alcoholic beverages (mostly wine) played a pivotal role in their culture. As the Sumerians did before, they offered wine to the deities but, they also used it as medicine, in rituals, for recreation, and even as an international trade commodity. Particular was the case of the *symposia*, which considered the perfect expression of Hellenic culture. It consisted of a banquet followed by the consumption of large quantities of wine. A large number of texts by classical philosophers have survived from the Hellenic period, many of which speak of the benefits of drinking wine, as well as habits and regulations associated with it. Quoting Panyasis (Yongue, 1854):

> *Wine is like fire, an aid and sweet relief,*
> *Wards off all ills and comforts every grief,*
> *Wine can of every feast the joys enhance,*
> *It kindles soft desire, it leads the dance.*
> *Think not then, childlike, much of solid food,*
> *But stick to wine, the only real good.*
> *Good wine's the gift which God has given*
> *To man alone beneath the heaven;*
> *Of dance and song the genial sire,*
> *Of friendship gay and soft desire;*
> *Yet rule it with a tighten'd rein,*
> *Nor moderate wisdom's rules disdain;*
> *For when uncheck'd there's nought runs faster,*
> *A useful slave, but cruel master.*

Taking this text as an example, it is possible to infer not only the high esteem in which wine was held, but also how the lack of control over its intake was already quite well know (Yet rule it with a tighten'd rein), a lack of control that could lead to future problems (A useful slave, but cruel master); in fact, there are contemporaneous texts that warn about the sickness subsequent to an episode of binge drinking and how to cure and avoid it.

In contrast, the next great Mediterranean civilization, ancient Rome, established itself as a sober society, prohibited the Bacchic ceremonies, in which people congregated to get drunk. However, this relationship with alcoholic beverages gradually changed as the master of winemaking soon became standardized. The conquering of new lands encouraged both production and exportation of wine resulting in surpluses, which led the Roman culture to adopt Greeks rituals for consuming wine.

The emergence of monotheistic religions represented a paradigm shift. For Christianity, which represent the world's largest religion with about 2.2 billion followers in 2010 (Hackett et al., 2017), wine is far beyond God's offering since it represents the transubstantiated blood of the son of God. Furthermore, a great deal of references to wine and vineyards appears in their holy writings and the eucharist (an act of Christian celebration) centres around the sharing of bread and wine. The other major religion in terms of numbers of followers today, Islam, currently holds a strong position against alcohol, but in the Koran, the references to alcoholic beverages are not consistent, with verses both extolling its benefits and others outlining rules on the prohibition of drinking it (Meri, 2018). Nonetheless, some authors report that these rules were frequently broken during certain periods of Islamic history, hence there are classical Islamic medical treatises describing the pernicious effect of heavy drinking and several references in classical Arabic literature (see Figure 2).

Figure 2. Representation of a festive scene in which music is played and wine is drunk. Wine and music, miniature of Maqamat de Al-Hariri (Source: Austrian National Library, Vienna).

In view of the above terse overview of the history of alcohol, it can be safely assumed that drinking behaviour has transcended civilizations and cultures even though in some cases the consumption of alcoholic beverages has been regulated or even prohibited. This rich history of alcohol consumption has motivated scientists to try to unravel the underpinnings of this apparent natural human inclination toward its consumption.

For those interested in learning more about the history of alcohol, a detailed and expanded version of this introduction can be found in the book “Drink: a cultural history of alcohol” by Iain Gately (Gately, 2008).

2. Current use and abuse of alcohol

The fact that there is a long history between humans and alcohol does not mean this relationship is confined to the past. According to the 2018 Global Status Report on Alcohol and Health (Hammer et al., 2018), around 2.3 billion people aged 15 and over are current drinkers, with a total-pure-alcohol-per-capita consumption of 6.4 litres in 2016 (a value that has increased by 0.9 points in 10 years). Moreover, the prevalence of Heavy Episodic Drinking (HED: a particularly pernicious type of alcohol use defined as the consumption of ≥ 60 grams of pure alcohol on at least one occasion at least once per month) is estimated to effect 18.2% of the global population, with 5.3% of death worldwide (3.9% in the case of Spain) (Donat et al., 2021) attributable to the harmful use of alcohol.

On the national level, in light of the latest data available from the Survey on Alcohol and Drugs in the General Population in Spain (Observatorio Español de las Drogas y las Adicciones, 2021), the situation is equally worrisome (see Figure 3). In 2021, alcohol was the most widely consumed psychoactive substance, with 77.2% of the people aged 15 and over admitting to having consumed it in the previous 12 months (93% if asked about having consumed alcoholic beverages at some time in their life), and nearly 1 in 5 acknowledging having gotten drunk in the same period. In fact, it is estimated that 1.300.000 people aged 15 – 64 have risky alcohol consumption.

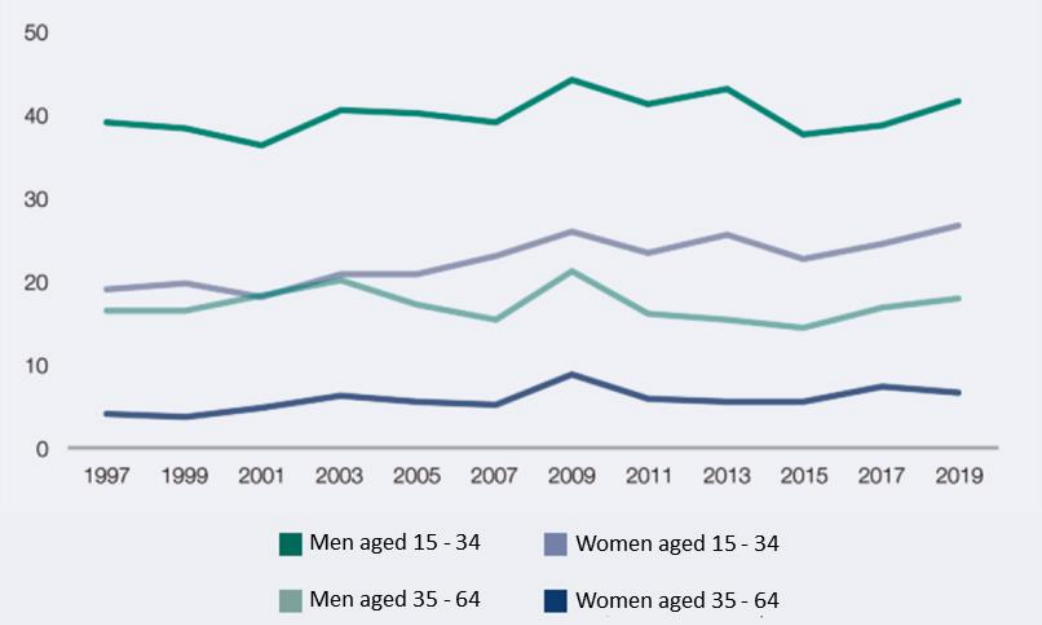

Figure 3. Evolution of the prevalence of drunkenness episodes in the last 12 month as a function of age and sex, in people aged 15 – 64 from 1997 to 2019 in Spain (Source: Observatorio Español de las Drogas y las Adicciones, 2021).

Regarding those aged between 14 and 18, alcohol is the psychoactive substance that they perceive as the least dangerous, hence it is not surprising that nearly half of the student population admit having had a binge drinking episode (BD; i.e. drinking 4 – 5 alcoholic drink in less than 2 hours) in the last year (National Institute on Alcohol Abuse and Alcoholism, 2004), which is associated with direct negative social, physical, academic, or sexual consequences (Adan et al., 2016; Kuntsche et al., 2017; Wicki et al., 2018), as well as long term effects like an enhanced tendency to exhibit an alcohol-use disorder (AUD) (Rial et al., 2020).

These high consumption rates have a plethora of both health and social consequences. Worldwide, it is estimated that alcohol use was the seventh leading risk factor in deaths and disability-adjusted life-years (DALYs) in 2016, being linked to 60 acute and chronic diseases; and contrary to popular belief, there is no level of consumption that does not incur a health threat; in other words, the only way to reduce any health loss associated with alcohol consumption is abstinence (Griswold et al., 2018).

The social cost associated with alcohol consumption, including the direct (i.e., medical healthcare) as well as indirect cost (e.g., loss of work productivity, injury, mortality, etc) (Axley et al., 2019), has been calculated to be at approximately 125€ billion in the European Union in 2003 (Laramée et al., 2013), and, regarding the United States, excessive drinking cost nearly $250 billion in 2010, which equates to a per capita cost of $807 (Sacks et al., 2015).

It is important to highlight that alcohol use is driven not only by neurobiological and contextual factors (Stanesby et al., 2019), but other conditions too. For instance, recent studies warn of comorbidity among BD and binge eating (BE) (Ferriter & Ray, 2011), which both involve the ingestion of large amount of alcohol or food on a single and brief occasion, usually associated with a feeling of loss of control (Dingemans et al., 2017).

It has been reported that the consumption of highly palatable foods (which are prominent in BE episodes), activate similar brain regions as those of drug abuse (Blum et al., 2017; Volkow et al., 2011), thus it is not surprising that, individuals exhibiting a heightened preference for very sweet sucrose solutions have been shown to have an increased risk of BE disorder (Goodman et al., 2018), which is a phenotype also related to AUD (Braun et al., 2021). Moreover, both binge behaviours appear to typically begin

in late adolescence or early adulthood, with comparable negative consequences such as physical problems, poor academic performance or social and psychological fallouts (for a review see (Ferriter & Ray, 2011).

3. Alcohol's claims to fame.

Despite the extolled organoleptic properties of some alcohol beverages and their multiple gastronomic, cultural and ceremonial uses throughout history, the reason behind alcohol (hereafter referred to as ethanol) consumption cannot be fully understood without looking at its psychoactive properties.

Ethanol is a volatile, flammable and colourless liquid (at room temperature), with antiseptic, disinfectant, toxic and pharmacological properties. The ethanol molecule (CH_3 - CH_2 - OH), whose three-dimensional model can be seen in figure 4, is a relatively simple and small organic chemical compound which is naturally produced by the fermentation of sugars by yeast (NCBI, 2022).

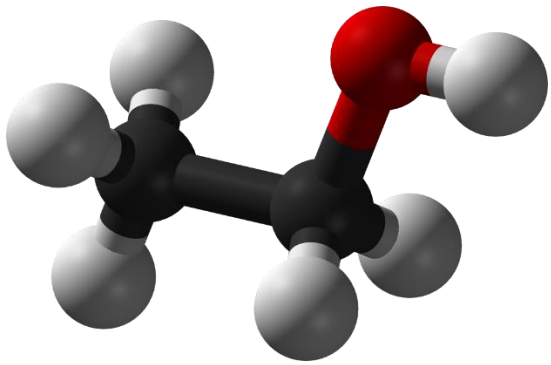

Figure 4. Schematic representation in 3 dimensions of the ethanol molecule. (Source: Public domain image)

Once swallowed (the most common route of administration) ethanol is absorbed chiefly via intestine walls, quickly reaching the cardiovascular system, where it is distributed, diluted in water, throughout the body (Pohorecky & Brick, 1988) reaching highly vascularized tissues expeditiously. One of these particularly exposed tissues is the brain, since the Blood-Brain barrier is highly permeable to ethanol, thus it allow blood and brain ethanol concentrations to correlate significantly.

Both desirable (e.g., anxiolytic, anti-inflammatory and immunomodulatory) and harmful (e.g., toxicity and related disease) (Birková et al., 2021) effects of ethanol have been a pivotal subject of study for scientists (see le Daré et al., 2019), who have employed bottom-up and top-down approaches in order to unravel its intricacies. Furthermore, ethanol is a non-specific drug with acute and long-term multilevel effects (Egervari et al., 2021) and, on top of that, the wide range of effects differs between subjects depending on

a variety of factors like sex, age, genetic background and physiological and environmental characteristic. A brief review of some of these actions can be found below (for an extended review see Abrahao et al., 2017; Nutt et al., 2021):

3.1. Neural molecular targets.

Ethanol's characteristic lack of specificity means that it effects several proteins in the central nervous system (CNS), in some cases through specific ethanol-binding sites, but, in some other, as a result of complex and non-specific interactions that remains unclear. Bottom-up approaches have identified the following direct targets:

- Alcohol dehydrogenases (ADHs): one of the best examples of studied ethanol-target molecules since it is the group of enzymes responsible for the two-step metabolism of ethanol that occurs mainly in the liver (Mackus et al., 2020). Its presence in the brain was confirmed in the studies of Raskin & Sokolof (Raskin & Sokoloff, 1968) and a large body of evidence has related it to both acute effects (Chan & Anderson, 2014) and ethanol-induced pathologies, as well as AUD (Crabb et al., 2004).
- Gamma-Aminobutyric acid receptor type A ($GABA_AR$): Is a cys-loop ligand-gated ion channel (LGIC) which mediates fast synaptic transmission. This protein contains a high affinity ethanol site located in the extrasynaptic δ subunit (Olsen, 2018). Years of extensive research has linked $GABA_AR$ to AUD and to both acute and chronic actions of ethanol in a brain-region-specific manner. In simple terms, ethanol exposure unbalances the equilibrium between the main inhibitory and excitatory neurotransmitters (GABA and glutamate respectively; see figure 5) which is related to sedative, anxiolytic, reinforcing (Ding et al., 2015), motor impairment and withdrawal effects of ethanol (Olsen & Liang, 2017; Roberto & Varodayan, 2017).

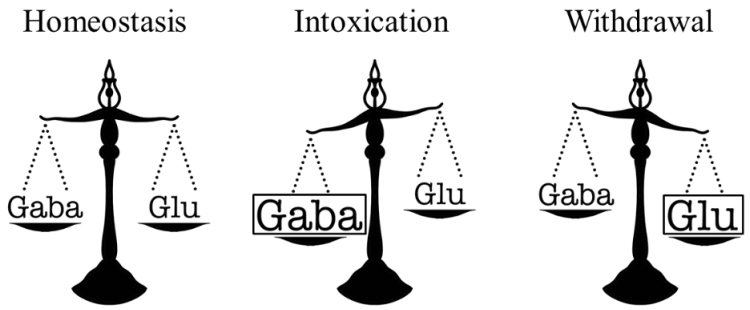

Figure 5. Representation of the misbalance between GABA and Glutamate (Glu) during ethanol acute intoxication and withdrawal state.

· Glycine receptors (GlyRs): Another of the LGIC-type receptors, which produces hyperpolarization and inhibition of neural signalling when glycine, its main agonist, binds to it. The ethanol binding site in this receptor remains unclear but in vitro studies indicate an action site located in the α1 subunit. In vivo studies in rats have demonstrated that GlyRs are involved in ethanol-reinforcing effects via ethanol-induced dopamine (DA) release (Söderpalm et al., 2017).

· Nicotinic acetylcholine receptor (nAChR): An LGIC-type receptor which allows cations to flow into the cell when the endogenous ligand acetylcholine binds to it. Several studies demonstrate that ethanol modulates nAChR function in a subunit-specific manner, but the structural basis of the direct interaction between ethanol and this receptor are uncertain. However, pharmacological and genetical approaches have linked nAChR with much of ethanol's behavioural responses, inter alia, the onset and frequency of the use, reward, sedation, consumption and locomotor activation (Miller & Kamens, 2020).

· N-methyl-D-aspartate receptor (NMDAR): considered a family of receptors due its diversity in subunits composition which leads to different properties. Once activated, the NMDARs allows cations to flow into the cell. Ethanol inhibits the activation of the NMDAR by interacting with different transmembrane domains of the GluN2A and GluN2B subunits, but the precise mechanism is still unknown (Naassila & Pierrefiche, 2019). Among the behavioural and pathophysiological effect related to the ethanol-induced inhibition mediated by NMDAR (and GABA), its implications in neuroapoptosis in the developing

brain leading to memory and cognitive impairments are noteworthy (Lotfullina & Khazipov, 2018; Mira et al., 2020).

- 5-Hydroxytryptamine Receptors Subtype 3 (5-HT_3R): Another receptor of the LGIC family which is widely known for its role in the brain-gut axis. Small alcohols (like ethanol) act as positive allosteric modulators with a binding cavity located between the M3 and M1 helices in the neighbouring subunits in the interface between extracellular and transmembrane domains (Gibbs & Chakrapani, 2021). 5-HT_3R seems to have a role modulating GABAergic and dopaminergic synapses, despite the low density of 5-HT_3R in mesolimbic areas. Its role in ethanol actions will be further depicted in following sections.
- Large-conductance Ca^{2+}-activated K^+ channel (BK): It seems appropriate to give the function of the ethanol-recognition site to the slo1 protein of the BK channel; however, ethanol exposure could produce potentiation, inhibition and refractoriness depending on different factors such as the type of exposition (acute/repeated) or the subunit composition of the channel itself. Even though the detailed mechanism of ethanol tolerance still under investigation (specially in mammals), a pivotal role has been attributed to BK in both acute and chronic behavioural tolerance to ethanol (for a review see Dopico et al., 2016).
- G-protein-coupled inwardly rectifying K^+ channel (GIRK): With an ethanol binding site proposed in Leu246 in the cytoplasmic regions (Toyama et al., 2018), GIRK channel regulates neural excitability. In the presence of ethanol, the GIRK channel changes to an open conformation increasing K^+ permeability and resulting in hyperpolarization of the membrane. Several behavioural effects of ethanol-like analgesia and acute withdrawal severity have been linked to GIRK channel by using animal models (Mayfield et al., 2015).

3.2. Ethanol-sensitive neurons.

The effects that ethanol exerts on the function of ethanol-sensitive proteins in the CNS (some of them depicted above) cause changes in the polarization of the membrane potential, modifying the excitability of certain neurons and, eventually, the synaptic plasticity and transmission. In the following paragraphs, a succinct review of ethanol's effects on these neurons are given (for an extended review see Abrahao et al., 2017):

· Dopamine neurons: Mainly located in substantia nigra (SN) and ventral tegmental area (VTA) with projections to hippocampus (HPC), striatum (STR), prefrontal cortex (PFC), nucleus accumbens (NAc) and amygdala (AMG). The role that dopamine (DA) plays in substance use and abuse has been broadly studied. As a result, locomotor and reward properties have been attirbuted to it. Put simply, DA neurons are responsible for the learning mechanism (among other functions), through which an addictive behaviour is implanted - a process that depends on how those neurons fire in response to a stimulus, leading to the development of long-term depression/potentiation (LTD/LTP) of certain synaptic transmissions (Wise & Jordan, 2021). Regarding the effect that ethanol exerts over this system, acute ethanol increases both burst and pacemaker-like firing of DA neurons, and increases extracellular DA levels as much in VTA as AMG, NAc (especially in the medial shell region) (Buck et al., 2021), PFC and STR (Dahchour & Ward, 2022), although chronic exposure and overall withdrawal trends decrease in DA transmission, an effect that seems to be related to the opioid system. Interestingly, the enormous heterogeneity of DA neurons (some with the capacity to release other neurotransmitters in addition to DA) (Trudeau et al., 2014) causes particular subsets of them to be especially sensitive to ethanol or to respond in seemingly opposite directions (Doyon et al., 2021).

· GABAergic neurons: As mentioned before, GABAergic cells constitute the primary inhibition system, and they interconnect regions all over the brain. There are a wide range of GABAergic neuron types (for a comprehensive review see Melzer & Monyer, 2020). As one of GABAergic transmission's main functions is the coordination of different brain areas and information processing, ethanol's effects seem to be region-specific, with enhanced GABA release (thus inhibition) in regions like cerebellum (Valenzuela & Jotty, 2015), basolateral amygdala (BLA) (R oberto et al., 2021) and VTA (Roberto & Varodayan, 2017), but, interestingly, ethanol also inhibits the firing of particular types of GABAergic midbrain neurons (Adermark et al., 2014). Furthermore, chronic ethanol exposure brings about changes in GABAergic transmission, which occur depending on the paradigm of exposure (Roberto & Varodayan, 2017). It is worth noting that the final behavioural output caused by the effect that ethanol has on ethanol-sensitive neurons is the result of the interaction between different systems. An example of

this is the role that medium spiny neuron (MSN) plays in regulating reward and ethanol-seeking behaviour (Hong et al., 2021), since MSNs are a subtype of GABAergic neurons that exhibit dopamine receptors, which are also hyperpolarized in the presence of ethanol.

· Glutamatergic neurons: Glutamate (Glu) is the main excitatory neurotransmitter in the CNS, and it is ubiquitous throughout the brain, as it is particularly important in processes such as plasticity, learning and memory. It has been shown that extracellular levels of Glu increase in a dose-dependent manner in NAc as a consequence of the exposure to ethanol (Buck et al., 2021). Moreover, glutamatergic transmission is also promoted in other brain regions like BLA, HPC, PFC and VTA, even at low/moderate doses, and many preclinical and clinical studies have linked AUD to changes in expression levels of Glu-related genes (for a review, see Bell et al., 2016)

· Cholinergic interneurons (ChI): These typically tonically active neurons located in the STR receive GABAergic, glutamatergic and dopaminergic afferents from the cortex and thalamus and have many long-branched axons which allow them to interact with MSNs as well as GABAergic interneurons and other ChIs, hence they seem to have a key modulatory role in striatal microcircuits (Abudukeyoumu et al., 2019). Acute ethanol exerts a complex effect on STR. With regards to ChIs, ethanol decreases the firing rate of these neurons by the inhibition of NMDA and AMPA receptors a process which has been related to ethanol addiction and the motivation to consume (Clarke & Adermark, 2015). Recent studies assessed the effect of chronic ethanol drinking in rats on ChIs have found a reduction in the thalamic excitation of the dorsomedial STR ChIs, which could lead to ethanol-induced impairment of cognitive flexibility (Ma et al., 2022).

· Other cells: Although most of the research done on the effect of ethanol on CNS has been done on neurons, glial cells are also directly or indirectly affected by ethanol due to their involvement in toxicity processes. An example of this is the modulation of microglia activation due to ethanol throughout toll-like receptors (Henriques et al., 2018).

3.3. Ethanol-related systems.

At this point, the reader may have an idea of the convoluted action of ethanol in the brain; however, other systems that modify the behaviour of ethanol consumption and/or are influenced by it are well-known (Koob et al., 1998), although the molecular mechanism behind that interaction remains unclear.

- Opioid system: This is a system consisting of several endogenous peptides that act as neurotransmitters which exert their effect in the CNS through four different receptors. Its role in addiction and drug- and food-reward have been widely studied; however, due to the complex nature of the system, unravelling the precise function of each one of its components is a goal yet to be reached (Conway et al., 2022). Nonetheless, the efforts made in this field have facilitated the identification of certain endogenous opioid receptors in which are involved in addiction, such as Dynorphin (DYN) and κ-opioid receptor (KOR) (Karkhanis et al., 2017). The DYN/KOR system has been shown to be upregulated after acute and chronic ethanol exposure and KOR antagonism seems to reduce both ethanol intake (being more effective at high levels of consumption) and negative effects associated with chronic ethanol exposure and withdrawal (Anderson & Becker, 2017; Rose et al., 2016). Interestingly, pharmacological modulation of μ-opioid receptor (MOR) in NAc shell via microinfusions produce either increased (with the MOR antagonist CTOP) or decreased (with the MOR agonist DAMGO) ethanol intake in alcohol preferring rats, but no effect was found with drugs that target KOR (Uhari-Väänänen et al., 2016), showing that modulation of ethanol intake through opioid receptors could be region and receptor specific. Conversely, previous similar approaches with DAMGO infusions in NAc (Zhang & Kelley, 2002) resulted in an increase in ethanol intake. Likewise, δ-opioid receptor (DOR), has also been found to interact with ethanol (Alongkronrusmee et al., 2016) through changes in expression levels and ligand affinity, and agonism and antagonism of DOR could reduce ethanol self-administration in rodent models. Furthermore, current findings (Bellia et al., 2020) show that rats selectively bred for high and low ethanol intake, under conditions of chronic ethanol exposure, exhibit differences in opioid-system-related-mRNA levels in PFC and NAc. It has also been shown that enkephalin (an endogenous ligand with high affinity to DOR) levels increase after chronic ethanol exposure (Chang et al., 2010). Due to

the promising role the opioid system could play as a therapeutic target, recent studies (Zhou et al., 2022) assessed novel pharmacological strategies in order to reduce conditions associated with ethanol consumption like withdrawal, and the treatment with the combination of KOR and MOR antagonists was found to be effective.

· Serotoninergic system: Since its discovery in the 1950s, a plethora of research has shown that this system is involved in higher mental function as well as pain, motor activity, and regulation of autonomic processes (Olivier, 2015). Serotonin (5-HT) in the CNS is synthesized primarily in the Dorsal Raphe Nuclei (DRN) and exerts its function through fourteen different receptors (5-$HT_{1A, 1B, 1D, 1E, 1F; 2A, 2B, 2C, 3, 4, 5A, 5B, 6 and 7}$), and it is reuptaken by the serotonin transporter (SERT) in the synaptic cleft after its release. With respect to ethanol, acute administration produces an increase in extracellular 5-HT levels, whereas chronic exposure has the opposite effect (Sari et al., 2011). Furthermore, a large body of evidence relates 5-HT system to the regulation of ethanol-related behaviour like intake, reward, preference and dependence, since 5-HT projections from the DRN innervates reward brain regions such AMG, NAc, PFC and VTA (Sari et al., 2011). For instance, a functional polymorphism in SERT gene that produces a hyperfunctional SERT has been related to early-onset alcoholism, since there are lower basal 5-HT levels in the synaptic cleft, thus homozygote carriers have less negative side effects and provoke stronger cravings. On the other hand, genetic or pharmacological manipulations that promote 5-HT levels in the synaptic cleft usually led to a reduction in the consumption of ethanol (Marcinkiewcz, 2015). The role 5-HT_3R plays in ethanol dependence must be highlighted since, as stated above, ethanol directly binds to this receptor, and also has a modulatory role in the increased mesolimbic DA activity associated with drugs abuse (Engleman et al., 2008). Historically, 5-HT_3R in AUD has receive an extensive research interest (Grant, 1995) in view of its rich pharmacology with selective and nonselective agonists and antagonists, the latter having a proven capability of reducing ethanol self-administration and/or its associated effects (Ding et al., 2015; Engleman et al., 2008).

· Endocannabinoid system: Much more recent is the history of this system since it was discovered in the late 1980s. It is composed of two receptors

(CB_1R and CB_2R). These consist of several arachidonic acid-containing lipids like anandamide and 2-arachidonoylglyerol that act as endogenous ligands, and the precursors and enzymes related to them. An increasing number of functions have been related to the endocannabinoid system, of which pain, learning, emotional state, stress reactivity and ethanol reward properties are worth highlighting (Kunos, 2020). The latter seems to be mediated by the CB_1R regulation of VTA-DA neurons, given that as acute ethanol increases DA, is released in a CB_1R-dependent manner by these neurons, and the genetical or pharmacological blockade of CB_1R prevent this DA release. Supporting the implication of CB_1R in ethanol consumption behaviour, studies of CB_1R expression have shown that strain rodents with innate predisposition to ethanol self-administration display lower CB_1R expression levels than strains with less preference for ethanol. Chronic ethanol exposure also reduces CB_1R levels and function, which seems to have implications for ethanol tolerance, dependence and withdrawal. More impressive are the cross-tolerance effects of ethanol and Δ^9-THC (Delta-9-tetrahydrocannabinol – an agonist of CB_1R which is the major psychoactive component of *Cannabis sativa*). There are synergetic interactions when the two compounds are co-administered and there are similar cognitive deficits after acute or chronic exposition to both (Basavarajappa, 2019). Furthermore, rats selectively bred for ethanol preference shows increased sensitivity to CB_1R agonist, thus demonstrating shared mechanisms between ethanol and cannabinoids (Hauser et al., 2020).

· Neuropeptides: This diverse group of aminoacidic molecules act as neuromodulators and neurohormones. They play a pivotal role in neuronal activity, hence some of them have been related to ethanol effects (Genders et al., 2020). Chronic ethanol exposure has been proven to increase the levels of neuropeptides such as galanin, orexin (Genders et al., 2020), corticotropin-releasing hormone (CRH) (Agoglia et al., 2020), neuropeptide S (during withdrawal) (Cannella et al., 2022) in regions related to ethanol seeking and consumption. It has also been proven to decrease neuropeptide Y (Robinson & Thiele, 2017) in such regions. Furthermore, pharmacological or genetic modulation of the mentioned neuropeptides produce changes in ethanol-related behaviours. Ethanol also alters oxytocin system (Ryabinin & Fulenwider, 2021) since it inhibits

its production and release, and oxytocin administration seems to reduce ethanol intake. Nevertheless, disparities in recent studies have produced a lack of consistency in such affirmations. Regarding cholecystokinin (CCK), the most abundant neuropeptide in the mammal brain, has been proposed as a promising target for the treatment of AUD, since CCK has been shown to act as intensity controller of neurotransmitters such as 5-HT, DA, GABA, Glu, orexins and oxytocin with proven implication in ethanol effects and consumption (Ballaz et al., 2021).

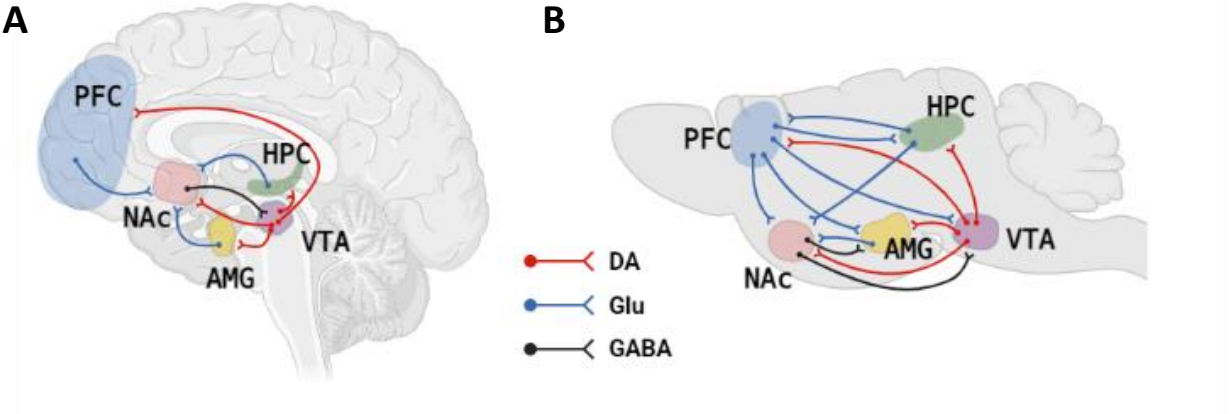

Figure 6. Schematic representation of some of the brain areas involved in drug reward and addiction. Abbreviations: AMG, amygdala; HPC, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

In order to give an intelligible overview of ethanol actions in the brain some of them have been detailed above in relation to their particular systems, since most of the studies are oriented in this way (a succinct representation can be found in figure 6). However, it must be highlighted that health and behavioural consequences of each type of ethanol intake must be understood as an outcome of the complex integration of all involved systems. Likewise, the interconnections between these systems means that the modulation of one of them, in order to assess any ethanol effects, has a mandatory action over others related systems. Altogether, this implies that research in ethanol field should take a holistic approach so as to successfully tackle the task of understanding the actions of ethanol and thus establish appropriate treatments.

4. Sigma-1 receptors and ethanol

Originally described as a new type of opioid receptor, the sigma receptors have now been characterized as singular types of proteins whose sequence of amino acids is unrelated to others known mammalian proteins. Currently, two subtypes of sigma receptors have been identified, of which sigma-1 receptor (S1-R) has been proposed as a promising therapeutic target for addiction, as well as other diseases (Cobos et al., 2008). More specifically, S1-R is an intracellularly located small chaperone (223 amino acids), highly conserved between species (Chu & Ruoho, 2016), which exhibits an ample pharmacology with several characterised agonists and antagonists (Vela et al., 2015). Exerting a modulating role in neurotransmission, in the CNS its presence is ubiquitous, and it can be found in brain areas related to substance use, abuse and addiction, such as AMG, HPC, NAc or VTA, among others (Cobos et al., 2008).

Although no direct interaction between ethanol and S1-R has been discovered, several studies demonstrate the influence of S-1R on ethanol-related effects. Locomotor-stimulating effects of ethanol have been dose-dependently attenuated by BD-1047 (a selective S-1R antagonist), and S1-R KO mice appear to be less sensitive to such an ethanol effect than WT mice (Maurice et al., 2003; Valenza et al., 2016). The rewarding properties of ethanol also have been related to S-1R by pharmacological modulation of the system in the context of the conditioned place preference (CPP) paradigm. Briefly, this paradigm is based on an experimental arena equipped with contextual cues, that, after repeated exposure to the drug, will be preferred to another neutral arena even when the drug is not provided. S1-R has been shown to modulate CPP induced by ethanol, with a blocking effect of BD-1047 and a facilitating effect of PRE-084 (a selective agonist) (Maurice et al., 2003).

Regarding ethanol intake, antagonism of S1-R has also been shown to reduce ethanol self-administration in 2-BC paradigm and chronic intermittent ethanol exposure in sP rats (Blasio et al., 2015; Sabino, Cottone, Zhao, Iyer, et al., 2009; Sabino, Cottone, Zhao, Steardo, et al., 2009), an effect that

is also shown in the consumption after withdrawal, when antagonist is administered preventively. Altogether, it seems that S1-R antagonism has the promising potential to be used in the development of new pharmacological strategies to treat AUD; however, studies with S1-R KO mice show that they exhibit a greater ethanol intake and preference

than WT pairs. This effect was not related to taste deficits (Valenza et al., 2016). KO mice might display a maladaptive behaviour for lacking S1-R systemically, thus results from KO studies must be analysed with care, and further research is needed to fully elucidate the effect of pharmacological S1-R modulation over ethanol effects.

5. Animal models of alcohol consumption

Despite the worthwhile information provided by *in silico, in vitro* and *ex vivo* research models, current progress in biomedical research would not have been possible without the use of animal research models, since they provide an invaluable tool for investigating behaviour associated with ethanol actions, its genetic and neurobiological factors and pharmacological strategies for its treatment (Bell et al., 2017).

Modelling ethanol intake in laboratory rodents has been an uphill battle due to the innate reluctance of mice and rats to self-administer ethanol, at least in amounts significant enough to produce toxic or pharmacological effects (Becker & Lopez, 2016). However, as the biomedical research into ethanol was progressing, several animal models arose that could be used to mimic a considerable number of factors related to the human behaviour of alcohol use (Bell et al., 2017). To establish valid rodent models for ethanol research various approaches have been taken: a) selective breeding or genetic manipulation, b) forced or passive administration c) voluntary or active consumption. All three are detailed below:

a) Several investigations have assessed the influence of genetics and the heritability AUD related factors (which is estimated to be 40 – 60%) (Ducci & Goldman, 2012), from family and twin studies to the latest genetic approaches with genome-wide association studies (Reilly et al., 2017). Regarding animal models, a well-established form of controlling and studying the influence of genetic heritance is selective breeding (Crabbe, 2014), which is a technique that has been used since the 1940s. Simply put, selective breeding procedure is based on offering water and an ethanol solution to the rodents over a period of time, and afterwards, having the males with higher ethanol consumption mate with the females that also have higher ethanol consumption while doing the same with the males and females that exhibit lower ethanol consumption. This method is replicated over the ensuing generations so that two separate strains are generated with different genetic backgrounds thus different ethanol-related behaviour. Some of these strains such as Sardinian alcohol-preferring (sP) and non-preferring (sNP) (Borruto et al., 2021) or low alcohol-drinking (UChA) and high-alcohol drinking (UChB) (Quintanilla et al., 2006) have been developed throughout several tens of generations and have been widely used in research. That being said, other researchers have successfully explored the possibility of short-term selection (just

a few generations) to create separate lines (Fernández et al., 2017). More recently, genetic engineering has allowed other approaches, with strain rodents with altered genes by using knockouts and knockin techniques (Mayfield et al., 2016). This has made it possible to target particular systems in order to assess their influence on ethanol actions. Although the value of the progress attributable to these models is indisputable, caution should be exercised when extrapolating the results and applying them to humans, since there are some discouraging examples that show discrepancies between human and genetic/selective-breeding-rodent models in effectiveness of a treatment or influence of certain systems on a disease (Borruto et al., 2021).

b) Forced administration allows researchers to give a certain dose of ethanol in a usually rapid and easy-to-perform way thus having greater control over the drug intake. Procedures like oral gavage, intravenous infusion or intraperitoneal administration have been used to study the effect of both acute or repeated administration of ethanol (D'Souza El-Guindy et al., 2010); however, these methods induce pain and stress in the animals and are barely ecological compared to human consumption. Another forced procedure is the alcohol vapor inhalation model, which consist of putting animals (intermittently or for a long period) in chambers where ethanol is insufflated, thereby allowing the assessment of the effect of chronic exposure to ethanol. Notwithstanding the proven efficacy of the vapor model in the development of addiction to ethanol (Vendruscolo & Roberts, 2014), the unorthodox method of administration, compared to that of humans, makes this model not ecological either. Finally, ethanol adulteration of diet or drinking water produces a sustained and chronic ethanol consumption, but the inability to feed or hydrate without the consumption of ethanol causes the rodents to dehydrate thus leading to behavioural and physiological consequences (Jeanblanc et al., 2019). The great limitation of all these models is the lack of voluntariness in consumption, therefore it is not viable to consider ethanol related factors as onset of seeking, consumption or motivation.

c) There is a diverse range of methods for inducing voluntary ethanol consumption in rodents despite their reluctance to self-administer ethanol and the problems inherent in oral administration (Meisch, 2001). The easiest way to assess rodents' voluntary ethanol consumption is through two-bottle choice paradigm (2-BC), which consists of simultaneously offering rodents two bottles (one filled with

water and the other with an ethanol solution). However, this method only results in considerable ethanol consumption in some scenarios (D'Souza El-Guindy et al., 2010; Fritz & Boehm, 2016; Jeanblanc et al., 2019): a) using rodent strains genetically predisposed to ethanol consumption and/or with previous ethanol experience/dependence; b) adulterating the taste of ethanol solution with sweeteners to promote intake; c) when the access to ethanol is limited, scheduling or providing it during the first hours of the dark cycle. An alternative method which provides more information about ethanol-related behaviours is self-administration operant paradigms, which are based on training rodents to obtain small quantities of ethanol through behavioural responses like pushing a lever (García-Pardo et al., 2017). Nevertheless, as in the prior method, the reluctance to intake (due to the aversive flavour/odour of ethanol) fuelled the implementation of techniques to improve self-administration, such as shorten operant sessions (Jeanblanc et al., 2019) or progressive substitution in the delivered solution of sucrose to ethanol (Pautassi et al., 2015).

Although the possibility of developing an animal model that mimics all the features of human addict behaviours is remote, the heterogeneous variety of models facilitates the successful recreation of some of its characteristics. Depending on the specific scientific question to be addressed, it is better to choose one model or another, perhaps even a combination. For example, forced administration models allow greater experimental control over ethanol doses than voluntary models such as 2-BC, which is why they have been extensively used to assess ethanol effects like toxicity and tolerance (D'Souza El-Guindy et al., 2010). However, studies on brain activity show different patterns of activation depending on the route of administration (Burnham & Thiele, 2017), which demonstrates the influence these routes have on the neurobiological effects of ethanol consumption. Furthermore, the enhanced ethanol intake in adult rats fuelled by ethanol exposure during adolescence only occurs when the ethanol intake during adolescence is voluntary; that is, it is ineffective if ethanol is forcibly delivered (Gilpin et al., 2012).

The importance of adolescence should be noted, as it is a critical period of development in which physical, cognitive and social changes related to drug reward occur in both rodents and humans (Bell et al., 2017; Spear, 2015, 2018). In fact, it has been proposed that the earlier the alcohol exposure, the greater the odds of AUD (Rial et al., 2020), and the first drunkenness episode is of upmost importance regarding predictive

cues of future problematic substance use (Vera et al., 2020), a behaviour that has been successfully replicated in rat models (Salguero et al., 2020). In this scenario, rodent models that mimic particularly pernicious drinking patterns like BD are essential to assessment of the mechanisms of alcohol early debut effect.

Regarding sex, females are historically underrepresented in pre-clinical biomedical research, an issue that is now being remedied due to the growing evidence of differences in ethanol-related behaviours/effect between males and females (Flores-Bonilla & Richardson, 2019; Hilderbrand & Lasek, 2018; Hitzemann et al., 2022).

Furthermore, there are other factors that might increase ethanol self-administration, thus they could be used as a way to assess ethanol actions and related behaviours. Several stress-induction models have shown their influence on ethanol intake (Camarini et al., 2018; Pucci et al., 2019), probably due to the anxiolytic properties of ethanol. Stimulant substances like amphetamine (Ruiz et al., 2018), nicotine (Haun et al., 2021; Tarren & Bartlett, 2017) and caffeine and caffeine/taurine mixture (Tarragon et al., 2021) also promote ethanol intake.

More impressive are the effects of dietary components of ethanol self-administration by rodents, which show striking differences between different commercially "standard" chow diets (Quadir et al., 2020), differences that may be related to isoflavone content (Eduardo & Abrahao, 2022). Moreover, it is known that concurrent and scheduled access to food and ethanol enhances ethanol intake (Meisch & Thompson, 1972). Nevertheless, driven by the increasing comorbidity of BE and BD disorders (Ferriter & Ray, 2011), recent studies assessing the influence of binging upon highly palatable foods on ethanol intake find that bingeing on a high-fat diet or sugar solution could promote ethanol self-administration (Avena et al., 2004; Blanco-Gandía, Ledesma, et al., 2017), which may be related to the high hedonic value of these diets (Brutman et al., 2020). Furthermore, BE behaviours in animal models have been shown to increase the release of DA in NAc (Rada et al., 2005) and cause withdrawal symptoms (Cottone et al., 2008), features that are shared with BD models.

Rationale and goals

As stated before, high alcohol consumption is a threat to the lifespan and wellbeing of the population (Griswold et al., 2018), but knowledge of this fact does not seem to motivate this population to significantly reduce its consumption. In the case of Spain, from 1995 to 2019, the prevalence of the consumption of alcoholic beverages at least once in a year has risen by 8.7%. Furthermore, the prevalence of BD has remained stable since 2003, which is particularly worrisome since it is a markedly hazardous pattern of drinking exhibited mainly by the youth population (Observatorio Español de las Drogas y las Adicciones, 2021). Of particular interest is the case of the comorbidity of BD with BE, which has been widely demonstrated in humans via different approaches (Birch et al., 2007; Munn-Chernoff et al., 2013, 2021). In light of this data, the need to continue with research into the mechanism underlying alcohol consumption cannot be emphasised enough.

Animal models of ethanol use remain one of the most valuable scientific implements for the study of ethanol-related behaviours and ethanol effects, especially those that show good face, construct, and predictive validity, namely, mimicking the consumption patterns and behavioural symptoms of ethanol intake, exhibiting equivalence with humans at neurobiological and neurochemical level in the causes and effects inherent in the consumption of ethanol, and responding to pharmacological treatment with proven efficacy in humans respectively (Ciccocioppo, 2012). Unfortunately, the models that resemble these features are scarce, especially those that mimic complex human behaviour or multi-substance-use situations like BE-BD comorbidity.

Despite the major impact on society and public health of AUD, there is currently a limited number of drugs that can be used in its treatment, probably due to the intricate biological mechanisms of ethanol actions (Akbar et al., 2018). The U.S Food and Drug Administration's (FDA) and the European Medicines Agency's (EMA) approved drugs are disulfiram, which inhibits ADH thus producing a severe toxic reaction in presence of ethanol; naltrexone, which is an opioid receptors antagonist, and it reduces ethanol intake and craving; and acamprosate, which has NMDA-R and Glu-R actions and seems to be effective when administered in conjunction with psychosocial support. Additionally, Nalmefene, another opioid receptor antagonist, was approved in 2013 for AUD by the EMA (López-Pelayo et al., 2020). However, these treatments have been shown to have only mild efficacy in AUD or only be effective in highly motivated individuals.

Some other drugs approved by the FDA and the EMA have the potential to be repurposed for AUD treatment due to their demonstrated effectiveness in preclinical or clinical trials. Among them some anticonvulsants, antipsychotics or antidepressants can be found (Akbar et al., 2018). Nonetheless, the lack of consistency in some cases and the early stage of investigation in others precludes them from application to AUD. Moreover, a recent meta-analysis study on clinical trials questions the effectiveness of some of the main drugs indicated for AUD treatment (Palpacuer et al., 2018).

There are, however, other compounds with promising efficacy in the treatment of AUD due to their effectiveness in preclinical research, among which is the S1-R antagonist family, although they are yet to be approved for medicinal use (Quadir et al., 2019).

Bearing in mind the lack of animal models for voluntary BD ethanol intake that mimic the human behaviour, in the present Doctoral Thesis the **first proposed goal** is to fill this void, at least partially, by putting forward a new self-administration BD model in rats. The procedure is based on the brief but repeated exposure to a large quantity of highly palatable sugary pellets that will lead to their consumption as BE, which will lead to high voluntary ethanol self-administration in the manner of BD.

In order to test the validity of this model, the **second goal** was to assess whether the consumption of ethanol occurred despite aversive consequences, so that the intake could be considered habitual or compulsive (Hopf & Lesscher, 2014; Radke et al., 2020). For this purpose, a procedure of adulteration of the ethanol solution with quinine was used in order to test whether ethanol self-administration became quinine-resistant at the end of the procedure.

The **third goal** was to corroborate whether the ethanol intake in this model was mediated, at least partially, by the opioid system thus the reduction of the ethanol self-administration by naltrexone was assessed (Guardia Serecigni, 2015). Whether the model exhibited predictive validity (i.e., translational value) was also assessed.

Fourthly, some preclinical studies show the bidirectional modulation of S1-R on ethanol intake (Quadir et al., 2019), thus the **fourth goal** was to evaluate the pharmacological treatment with agents that target S1-R on the ethanol intake in this model.

Finally, in continuing to further assess the effectiveness of S1-R modulation on ethanol intake, the **fifth goal** was to assess the effectiveness of the antagonism of S1-R on the ethanol intake in a rat model of ethanol self-administration during adolescence.

Material and Methods

1. Animals

The subjects were male and female Wistar rats purchased from Envigo Laboratories (Barcelona, Spain), Charles River (Les Oncins, France), or they were reared at the Instituto de Investigación Mercedes y Martín Ferreyra (INIMEC-CONICET-UNC; Córdoba, Argentina). The rats employed were approximately 70-80 days old at the beginning of the experiments on adult rats and 26-27 days old at the beginning of the experiment on adolescent rats.

Rats were housed in polycarbonate cage and maintained under a 12-h light/dark cycle (lights on at 6.00 am) in a room with constant humidity and temperature (50 – 60% and 21ºC). The adult rats were housed individually while the adolescents were housed in pairs. The experiments using adult rats were performed during the light time of the day-night cycle. With the adolescent rats, part of the procedure was performed at the beginning of the dark cycle.

The procedures carried out at University of Granada (Experiment 1 to 4) was approved by the University of Granada Research Ethics Committee (Protocol number 09/08/2019/138); similarly, the procedures carried out at INIMEC-CONICET-UNC (Experiment 5A and 5B, the latter carried out by the PhD student Agustín Salguero) complied with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals of NIH and were certified by the Animal Care and Use Committee at INIMEC-CONICET-UNC.

2. Reagents and instruments

Ethanol solutions (2, 6, 10, 14% w/w) were prepared by diluting 96% (v/v) food-grade ethanol (PanReac AppliChem, Barcelona, Spain) with tap water. The solutions were presented to rats in 150 ml anti-drip bottles (Classic Drinker de Luxe, Zooplus, Munich, Germany) in their home-cage.

To induce BE, rats were provided with dustless precision pellets (DPP; 45 mg each, nutritional profile 59.1% carbohydrate, 18.7% protein, 5.6% fat, 3.6 kcal/g; Bioserve, Femington, USA). Rats were exposed to DPP in an empty polycarbonate cage (42.5 x 26.5 x 15 cm) identical to their home-cage.

To assess whether the intake of ethanol was compulsive, solutions were adulterated with 0.01 – 0.3 g/L of quinine monohydrochloride dihydrate 90% (Sigma-Aldrich, Madrid, Spain).

The opioid antagonist naltrexone (Sigma-Aldrich, Madrid, Spain), S1-R antagonists S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) and BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (both supplied by Esteve Pharmaceuticals, Barcelona, Spain), and S1-R agonist PRE-084 (2-(4-morpholinoethyl)-1-phenylcyclohexane-1-carboxylate Hydrochloride) (also supplied by Esteve Pharmaceuticals, Barcelona, Spain) were dissolved in sterile physiological saline solution and administered subcutaneously (s.c.) at a volume of 5 mL/kg.

Locomotor activity was assessed in a 3-minutes Open Field (OF) test to assess the motor-stimulating effect of voluntary ethanol intake. The OF apparatus was a black methacrylate cube cage (60 x 60 x 60). The test was recorded and later analysed with ToxTrack animal tracking software (Rodriguez et al., 2018). Recognition memory was assessed via the novel object recognition (NOR) test. This consisted of a square-shaped arena (50 x 50 x 50 cm) that was explored by rats in three phases, which were recorded. In the first one (PD 46), rats were habituated to the empty area for 10 minutes. Two identical opaque glass flasks (objects A and A') were presented in familiarization phase (PD 47) for 5 minutes, and finally, the rats underwent a 5-minute testing phase (PD 48) where A or A' objects were replaced by one slightly transparent flask (object novel or B). Distance travelled in the habituation phase and time spent exploring the objects in familiarization and testing phase were measured (Salguero et al., 2020).

3. Procedures

Experiment 1 – From BE to BD model

In this experiment a total of 91 male Wistar rats, aged 70 – 80 days old and weighing 280g (± 37) at the beginning of procedures were used. The rats were divided into experimental groups according to the following factorial design: 2 (BE [i.e., 72 DPPs] or control eating condition [i.e., 6 DPPs]) x 5 (ethanol concentration: 0%, 2%, 6%, 10% and 14%), with 6 – 13 rats in each cell of the design.

In order to resemble consecutive episodes of BE (i.e., the expeditiously ingestion of high amount of palatable food) the instrumental successive negative contrast performed by Rosas et al., (2007) was adapted, in which rats had 30 seconds to ingest a high (12 DPP) or low (1 DPP) food reward. Aimed at inducing a BE ingestion, rats were given 3 minutes to eat 72 DPP (6 DPP for those rats in control groups), an amount that represented the ingestion of 11.66 kcal, which is a caloric intake similar to that provided in others BE paradigms (Cottone et al., 2012; Curtis et al., 2019).

Preliminary experiments indicate that the ingestion of 72 DPP in 3 minutes is an achievable task for adult rats, but only if the ingestion is performed quickly and without interruption. Typically, the rats increase the number of DPP they eat across sessions, and the BE pattern (i.e., effectively eating the 72 pellets in 3 min) is exhibited by most of the rats from the fourth DPP exposure session onwards (Figure 7).

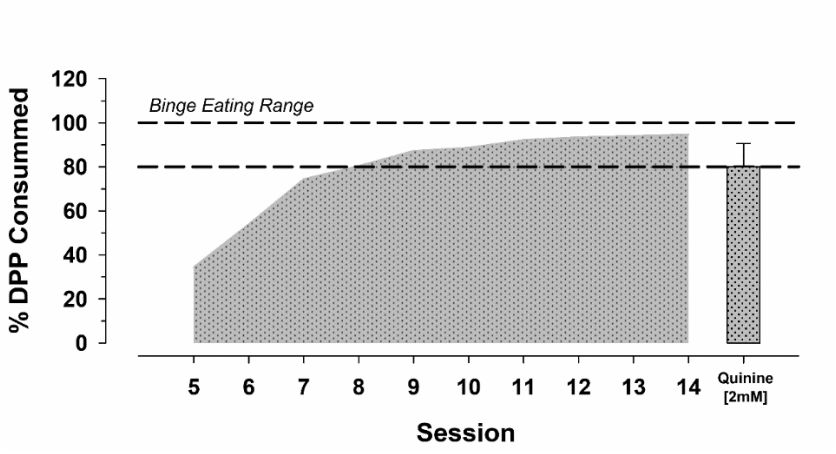

Figure 7. Percent of DPP consumed by the rats across sessions and when DPP was adulterated with a 2 mM quinine solution (as found in preliminary experiment from our lab). Dashed lines mark the percentage of DPP consumption in the Binge Eating range in our model.

It should be noted that once the consumption pattern of the DPPs offered in each session is established as BE, rats exhibit a mean intake greater than 80% of the available DPP, even when the pellets are adulterated with an aversive tasting quinine solution (2mM) (Figure 7).

The procedure began with a three-day period of mild caloric restriction so that the rat reached 82 – 85% of their body weight (sessions 1 – 3). On session 4, a habituation session was conducted as follows: rats were place in an empty polycarbonate cage for 3 minutes and then were returned to their home-cage, which was equipped at that time with two anti-drip bottles (one with ethanol solution and one filled with water, or two bottles of water, depending on the group assigned to each animal). 90 minutes after that, the two bottles were replaced by a regular bottle of water. Finally, rats were given 6 DPPs and rat chow in their home cages. The aim of this session was to familiarize the rats with the stimuli that would be presented during the subsequent sessions.

For 10 consecutive days (sessions 5 – 14), rats were weighed and exposed to a simulated BE episode or a control eating episode (i.e. 72 or 6 DPPs were offered in an empty polycarbonate cage for 3 min), depending on their experimental group. Immediately after, rats were tested in a two-bottle choice test (90 minutes long), in which they were presented, depending on the group, with two bottles of water or one bottle of ethanol solution (2, 6, 10 or 14% w/w) and one bottle of water. The relative position of the two bottles were alternated daily to avoid place preference effects.

Fluid intake was measured by weighing the bottles before and after each session. Fluid leakage was estimated by placing the same anti-drip bottles in an empty home-cage and subtracted from the measured fluid intake to accurately calculate ethanol intake (g/kg), percent preference of ethanol intake, overall liquid intake and water intake (g/kg). In between sessions, the rats were provided with *ad libitum* water and enough chow to maintain established body weight. A schematic representation of the procedure can be found in Figure 8.

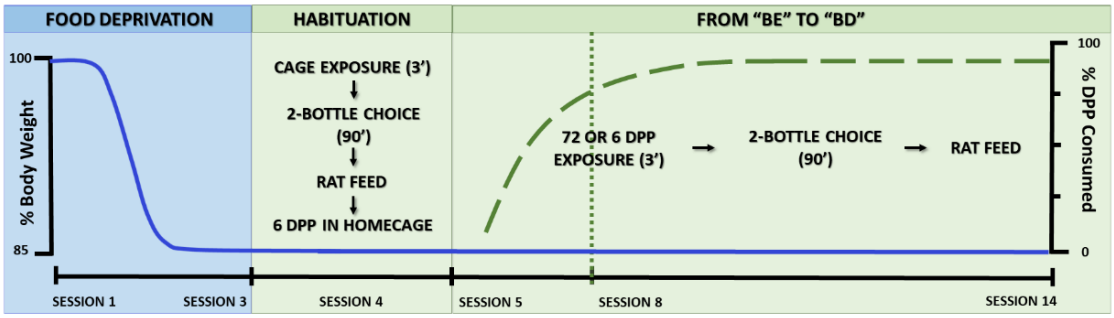

Figure 8. Schematic representation of body weight scores (solid line) and Dustless Precision Pellet (DPP) consumption (long dash line) exhibited by the rats during the experimental timeline of Experiment 1. The dotted line marks the beginning of the BE-like DPP consumption.

A cohort of 64 rats randomly selected from all groups performed an OF test where the total distance travelled was measured. The test, which lasted 3 minutes for each subject, was carried out immediately after sessions 12 – 14 and was conducted and recorded under identical conditions for each rat (50 dB, 63 lux, 11:00 – 12.00 am). The purpose of the OF test was to assess the potential stimulating effect of voluntary ethanol self-administration.

Finally, 11 blood samples of randomly selected rats (different from those which performed the OF test) were collected for blood ethanol level (BEL) determination. In the last sessions of procedure (12 – 14) and right after the 2-bottle choice test, about 200 µl of tail vein blood were drawn by making a small incision in the rat's tail with a surgical scalpel. BELs were determined in the toxicology facilities of the San Cecilio Hospital (Granada, Spain) using a headspace gas chromatograph (Perkin Elmer Clarus 580) equipped with a flame ionization detector (FID) and a headspace sampler (Perkin Elmer Turbomatrix 16). The injection time was 6 seconds with 4 minutes of pressurization. A polyethylene glycol (PEG) column (Elite-WAX 30 m × 0.53 mm i.d., 1.0 µm film thickness, PerkinElmer) was used. The temperatures for the column, the injector and the detector were set at 70 ºC, 150 ºC and 250 ºC respectively. Carrier gas flow was 30 ml/min of nitrogen. Samples were heated in the head space glass vials for 20 min at 60 °C for equilibration before injection. The internal standard n-propanol alcohol (25 µL) was added to the total blood sample (100 µL) with heparin, which was kept in a hermetically

closed vial at constant temperature (60 ºC). The vaporized alcohol from the sample was then inserted in the gas chromatograph.

Under the conditions of experiment 1, rats exposed to BE and 10% ethanol solution (and water) exhibited the greatest level of ethanol self-administration, therefore these conditions were replicated to perform the test to validate the model, namely, experiments 2 and 3.

Experiment 2 – From BE to BD model: intake despite aversive consequences

Alcohol consumption despite aversive consequences has been assumed as a sign of compulsive intake or habitual behaviour. One of the most widely used methods to model such aversive consequences in rodent models of alcohol self-administration is the adulteration of ethanol solution with the prototypical bitter component quinine (Hopf & Lesscher, 2014; Radke et al., 2020).

In order to address this aim, Experiment 2 replicated the procedures of Experiment 1, yet on sessions 12 – 15, the rats (70 days old, 341 ± 24 g at the beginning of the procedure) were divided into two groups. Those belonging to the quinine-adulterated ethanol solution group (n=10) were given 10% w/w ethanol contaminated with quinine (concentration: 0.01, 0.03, 0.1 or 0.3 g/L, sessions 12 to 15 respectively), whereas control rats (n= 10) received unaltered 10% w/w ethanol. In this experiment, only the BE condition was tested, and the duration of the two-bottle choice tests was reduced to 45 minutes.

Experiment 3 – From BE to BD model: assessing predictive validity

The predictive validity of an animal research model refers to its responsiveness to approved drugs for a certain medical condition (Ciccocioppo, 2012). Experiment 3 assessed whether the opioid antagonism throughout the FDA/EMA-approved drug naltrexone (Guardia Serecigni, 2015) inhibited BD after the exposure to the BE condition.

For this purpose, Experiment 3 replicated the procedures of Experiment 1, yet as in the case of Experiment 2, only the BE condition was tested, and all rats (n = 35; 70 – 75 days old; 295 ± 15g at the beginning of the procedure) were stimulated for 45 min with a

bottle of water and a bottle of 10% w/w ethanol. On session 12, 30 minutes prior to the 2-BC procedure, the rats were divided into 3 groups and received an acute dose of naltrexone depending on their experimental group (0, 1 or 10 mg/kg). The length of the two-bottle choice test was chosen to ensure that testing took place under the pharmacological actions of naltrexone.

Experiment 4 – From BE to BD model: modulation of S1-R

As stated before, S1-R modulation has been put forward as a promising therapeutic target for AUD (Quadir et al., 2019). S1-R antagonism has been shown to reduce ethanol seeking and intake in several research models (Blasio et al., 2015; Maurice et al., 2003; Sabino, Cottone, Zhao, Iyer, et al., 2009; Sabino, Cottone, Zhao, Steardo, et al., 2009). On the other hand, the administration of S1-R agonists seems to have a promoting effect over the motivational and rewarding effect of ethanol (Bhutada et al., 2012; Maurice et al., 2003; Sabino et al., 2011).

Given these precedents, Experiment 4 aimed to assess the effect of S1-R agonism and antagonism over voluntary ethanol intake in the BE-BD model. Specifically, 120 male Wistar rats (60 – 80 days) were exposed to the procedures conducted in Experiment 3 (i.e., all rats assigned to the BE condition and 10% – in procedures in which BD-1063 or PRE-084 were administered – or 6% (w/w) ethanol – in procedures in which S1RA were administered – in the 45-minute 2-BC test) but drugs were administered in accordance with the following designs: acute administration of the S1-R antagonists BD-1063 (0, 2, 8 or 32 mg/kg) or S1RA (0, 4, 16 or 64 mg/kg); or the S1-R agonist PRE-084 (0, 4, 8 or 16 mg/kg) on session 12, 30 minutes prior to the 2-BC procedure. Additionally, a preventive strategy in which the highest dose of S1RA was administered (64 mg/kg vs. vehicle) daily 30 minutes prior to the 2-BC procedure being evaluated.

Experiment 5 – BD in adolescence: effect of S1-R antagonism

Despite the promising effect that S1-R antagonism has shown, the influence of this drugs on ethanol intake in adolescence or in females remains unexplored (Quadir et al., 2019). This is particularly unfortunate since S1-R expression seems to be age-dependent (Moradpour et al., 2016), and several sex-related differences regarding drug use have

been reported in both preclinical (Flores-Bonilla & Richardson, 2019; Roth et al., 2004) and clinical studies (Erol & Karpyak, 2015). To fill this gap, Experiment 5 assessed the role of pharmacological antagonism of S1-R system in a model of binge-like ethanol self-administration in both male and female adolescent Wistar rats.

More accurately, 169 adolescent Wistar rats (80 in Experiment 5A and 89 in Experiment 5B, of which 40 in 5A and 39 in 5B were male) were employed in a paradigm of binge drinking as follows. The procedures began with a habituation session consisting of a 24h-2BC test (8% v/v vs. water) on postnatal day (PD) 27. Choosing this age as start point was not an arbitrary decision, since it has been reported that there is an equivalence between PDs 27 - 28 to 42 and PDs 46 to 59 in rats and early/mid adolescence and late adolescence in humans respectively (Bell et al., 2017; Burke & Miczek, 2014; Karanikas et al., 2013; Spear, 2015). For the consecutive 2 weeks (PDs 30 – 41) rats were exposed to a single bottle of 8% (v/v; sessions 1 and 2) or 10% (v/v; 3rd and following sessions) ethanol solutions (96% ethanol, Porta, Córdoba, Argentina) for 120 minutes, three times per week (Tuesday, Thursday and Saturday). The ethanol solutions were provided 15 minutes after lights out, namely, at the beginning of the 12h-night period. The day before each session, rats received 50 % of the water they usually consumed. A 2-BC test (8% v/v vs. water) was conducted 72 hours after the last binge session. The ethanol solution or water bottles were weighed before and after each session to calculate ethanol intake (g/kg), perfect preference of ethanol, overall liquid intake and water intake (ml/kg of body weight). A schematic representation of the procedure's timeline can be found in Figure 9.

In Experiment 5A, a 4 (S1RA dose: 0, 4, 16 or 64 mg/kg) x 2 (Sex) factorial design was employed (n = 10/ group). Rats were administered with an S1RA dose or vehicle 30 minutes before each binge session.

Experiment 5B replicated the procedure of Experiment 5A but a 4 (BD-1063 dose: 0, 2, 8 or 32 mg/kg) x 2 (Sex) factorial design was employed, with 7 – 10 rats per group. On PD 46 to 48, a cohort of 6 rats from each group were tested for short-term memory in NOR procedure. Additionally, 18 naïve (i.e., not exposed to ethanol) rats (8 males) were also tested in NOR procedures as a control.

Figure 9. Timeline depicting the procedures conducted to assess the effect of S1-R antagonism on ethanol self-administration in adolescent Wistar rats in Experiment 5.

4. Statistical Analyses

Preliminary results indicate that, under the DPP exposure protocol carried out in the BE-BD model, Wistar rats exhibit a BE behaviour (i.e. they eat most of the ration of DPP offered in the given time) from the eighth session onwards. Thus, in Experiment 1, the analyses were separately carried out for the acquisition of BE-like consumption phase (sessions 5 – 7) and BE-like DPP consumption phase (sessions 8 – 14). Ethanol intake (g/kg and %preference vs. water) and water intake (g/kg) were analysed via separated measure (RMs) analyses of variance (ANOVAs). Sessions 5-7 or 8-14 were the within-measures, whereas Ethanol concentration (0, 2%, 6%, 10%, 14%) and Eating condition (BE, CONTROL) were the between-group factors. Separate Pearson's time-moment correlation analyses assessed the association between the ethanol intake scores achieved by the rats and the distance travelled during the OF test or BELs measured on the day of sampling.

In Experiment 2 ethanol intake (g/kg) was analysed for sessions 11 (last session before ethanol was adulterated with quinine) to 15 via a RM ANOVA; whereas in Experiment 3 and in the acute approaches of Experiment 4, ethanol intake (g/kg) was analysed for sessions 11 (no administration of drugs), 12 (day in which the rats were administered) and 13 via a RM ANOVA. In the preventive strategy with S1RA in Experiment 4, ethanol intake (g/kg) was analysed following the procedure of Experiment 1, but S1RA dose (vehicle vs. 64 mg) was the between-group factor.

Finally, in experiment 5, the recorded intake in habituation session were analysed via Student's T test. Meanwhile, ethanol intake (g/kg) during the binge drinking sessions was assessed using RM-ANOVA, with Sex and Drug dose used as between factors and sessions (1 to 6) as within-measure. Fluid intake scores in the 2-BC test carried out after the BD sessions were also analysed via RM-ANOVAs, with Sex and Drug dose used as between factors. A relative discrimination index (Di; i.e., time spent exploring the novel object minus time spent exploring the familiar object divided by total exploration time) was calculated at the NOR test and analysed via ANOVA.

The partial eta-squared (η^2p) was used to report the effect sizes of the ANOVAs. Tukey's post hoc test was used to explore the significant main effects and significant interactions yielded by the ANOVAs and the differences between means were considered statistically significant when $p < 0.05$.

Results

Experiment 1 – From BE to BD model

In Experiment 1, a total of 7 rats were identified as non-responders to the BE protocol (i.e., exhibited a complete avoidance of the DPPs or the ethanol solution) and thus were excluded from the experiment.

During the BE acquisition sessions (i.e., sessions 5 – 7), the RM ANOVA for water intake (Figure 10, Panels A – B) revealed a significant main effect of Eating Condition ($F_{1,81} = 14.41$, $p < .001$, $\eta^2p = .15$), with the rats exposed to BE drinking more water than their CONTROL peers. The two-way interaction Session x Ethanol Concentration ($F_{8,162} = 4.43$, $p < .01$, $\eta^2p = .14$) was also significant. The post-hoc test revealed that, in session 7, the rats exposed to 10% ethanol drank significantly less water than those exposed to two water bottles (i.e., those in the 0% Ethanol Concentration group). With regards to water intake scores during the sessions 8 – 14 (those in which the rats achieve BE-like consumption of DPP; Figure 10, Panels A – B), the corresponding RM ANOVA revealed significant main effects of Ethanol Concentration ($F_{4,81} = 6.09$, $p < .001$, $\eta^2p = .23$) and Eating Condition ($F_{1,81} = 21.28$, $p < .001$, $\eta^2p = .21$), as well as a significant interaction between these variables ($F_{4,81} = 3.52$, $p < .05$, $\eta^2p = .15$). The post hoc test revealed significantly greater water intake in the BE – 0% group than in any other group, except for the BE – 14% group. Rats in the latter group drank significantly more water than their CONTROL peers and those rats given CONTROL and 6% or 10% ethanol. These results are depicted in Figure 10.

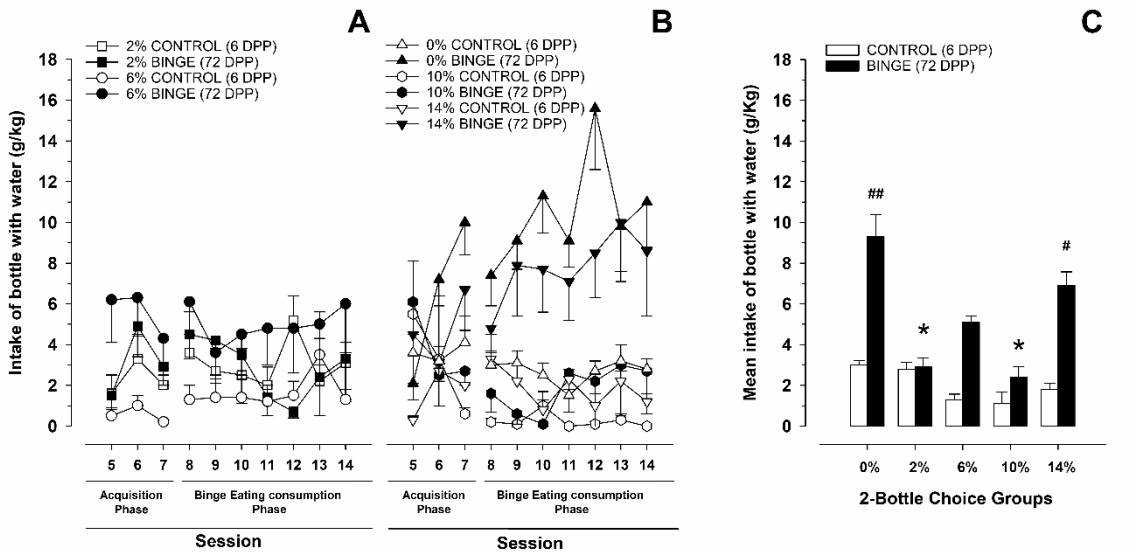

Figure 10. Panels A – B. Water Intake (g/Kg) in Wistar rats as a function of Ethanol Concentration (0, 2, 6, 10 or 14%), Eating Condition (BINGE or CONTROL DPP exposure) and Session. **Panel C.** To facilitate data visualization, this panel depicts the same data as A – B yet collapsed across sessions. Each point or bar and vertical line represent the mean ±SEM of the values obtained in 7–12 animals per group. (Panel C) Statistically significant differences between the values obtained in BINGE and CONTROL groups in each ethanol concentration: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; and between the values obtained in BINGE groups compared to 0% ethanol concentration: $*p < 0.05$.

The RM ANOVA for ethanol intake (Figure 11, Panels A – B) during sessions 5 – 7 revealed significant main effects of Session ($F_{2,114} = 14.95$, $p < .001$, $\eta^2p = .21$) and Ethanol Concentration ($F_{3,57} = 8.85$, $p < .001$, $\eta^2p = .32$), as well as a significant interaction between Ethanol Concentration and Eating Condition ($F_{3,57} = 4.29$, $p < .01$, $\eta^2p = .18$). The subsequent post hoc analyses revealed significantly greater ethanol intake in session 7, compared to the previous sessions, and significantly greater ethanol intake in rats exposed to BE and 10% ethanol than in the other groups, except for the CONTROL – 14% ethanol group. The RM ANOVA for ethanol intake in sessions 8 to 14 (see descriptive data in Figure 11) indicated significant main effects of Session ($F_{6,318} = 4.88$, $p < .001$, $\eta^2p = .08$), Ethanol Concentration ($F_{3,53} = 18.26$, $p < .001$, $\eta^2p = .51$) and Eating Condition ($F_{1,53} = 52.59$, $p < .001$, $\eta^2p = .50$). The ANOVA yielded significant two-way interactions between Session and Ethanol Concentration ($F_{18,318} = 1.99$, $p < .05$, $\eta^2p = .10$), Session and Eating Condition ($F_{6,318} = 3.14$, $p < .01$, $\eta^2p = .06$) and Ethanol Concentration and Eating Condition ($F_{3,53} = 5.02$, $p < .01$, $\eta^2p = .22$). The three-way interaction Session x Ethanol Concentration x Eating Condition was also significant ($F_{18,318} = 1.81$, $p < .05$, $\eta^2p = .09$; see Figure 11, Panels A – B). Tukey's post hoc test revealed that rats exposed to BE and given 6 or 10% ethanol drank more than their

CONTROL counterparts in sessions 10 – 12 or in sessions 9 and 11 – 14 respectively; furthermore, rats given BE and 10% ethanol also drank more ethanol than those given BE and 2% ethanol (sessions 8 – 14) or BE and 14% ethanol (sessions 9, 12, 13). Moreover, in session 8, rats given BE and 2% had a lower ethanol intake than those belonging to the BE – 6% group. No significant differences were found between rats given CONTROL Eating Condition, regardless of the volume of alcohol contained in ethanol bottle.

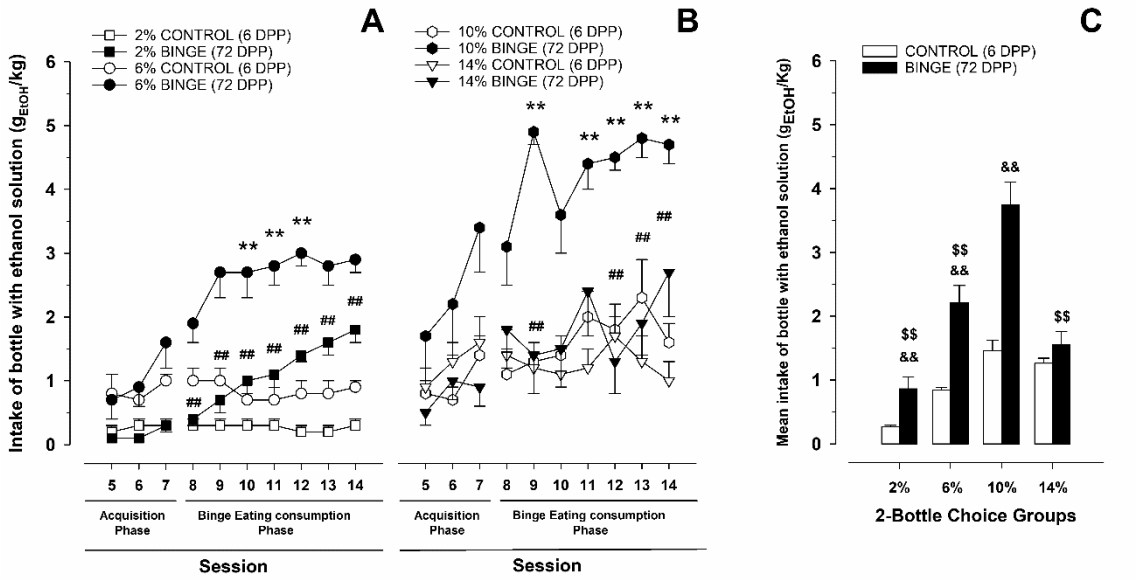

Figure 11. Panels A – B. Ethanol Intake (g_{EtOH}/Kg) in Wistar rats as a function of Ethanol Concentration (2, 6, 10 or 14%) and Eating Condition (BINGE or CONTROL DPP exposure) and Session. **Panel C.** To facilitate data visualization, this panel depicts the same data as A – B but collapsed across sessions. Each point or bar and vertical line represents the mean ±SEM of the values obtained in 7–12 animals per group. (Panels A-B) Statistically significant differences between the values obtained in BINGE and CONTROL groups: *$p < 0.05$; **$p < 0.01$; and between the values obtained in BINGE groups compared to 10% ethanol concentration: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. (Panel C) Statistically significant differences between the values obtained in BINGE and CONTROL groups in each ethanol concentration: $^{\&\&}p < 0.01$; and between the values obtained in BINGE groups compared to 10% ethanol concentration: $^{\$\$}p < 0.01$.

With regards to ethanol preference (Figure 12, Panels A – B) the ANOVA for sessions 5 – 7 yielded significant main effects of Session ($F_{2,104} = 4.83$, $p < .01$, $\eta^2p = .08$) and Eating Condition ($F_{1,52} = 9.01$, $p < .01$, $\eta^2p = .15$), and a significant interaction between Session and Alcohol Concentration ($F_{6,104} = 2.26$, $p < .05$, $\eta^2p = .12$). The post hoc tests on the significant main effects revealed significantly greater preference in session 7 than in the other sessions, and significantly lower preference scores in rats exposed to BE than in CONTROL rats. The post hoc tests conducted to explore the

significant two-way interaction did not reveal significant differences. The ANOVA conducted to analyze ethanol preference during sessions 8 – 14 yielded a significant main effect of Ethanol Concentration ($F_{3,50} = 6.74$, $p < .001$, $\eta^2p = .29$), as well as a significant interaction between Alcohol Concentration and Eating Condition ($F_{3,50} = 3.76$, $p < .05$, $\eta^2p = .18$), and between Session and Ethanol Concentration ($F_{18,300} = 1.75$, $p < .05$, $\eta^2p = .09$). The three-way interaction ($F_{18,300} = 1.95$, $p < .05$, $\eta^2p = .10$) was also significant. In session 9, the rats exposed to BE and 14% ethanol exhibited significantly lower preference for ethanol than those exposed to 10% or 6% (regardless of the consumption of DPP or lack thereof). This effect was also observed in session 10 for rats assigned to the BE – 10% ethanol group, and in session 12 for rats belonging to the groups BE – 2% ethanol or CONTROL – 10% ethanol.

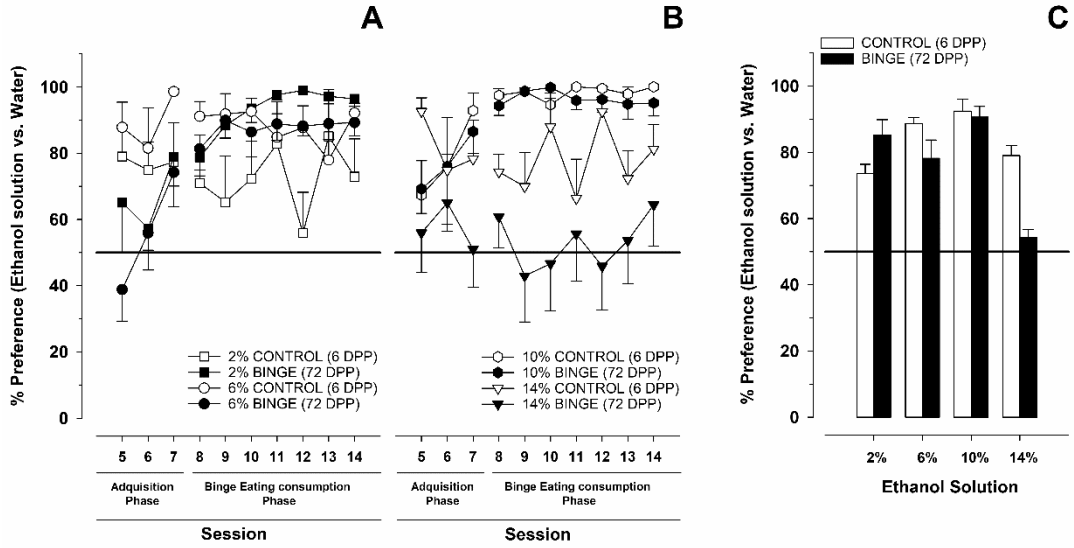

Figure 12. Panels A – B. Preference scores (percent ethanol preference vs. water) in Wistar rats as a function of Ethanol Concentration (2, 6, 10 or 14%) and Eating Condition (BINGE or CONTROL DPP exposure) and Session. **Panel C.** To facilitate data visualization, this panel depicts the same data as A – B but collapsed across sessions. Each point or bar and vertical line represents the mean ±SEM of the values obtained in 7–12 animals per group. A reference line has been set at 50% of preference, indicating that there is no preference at this value.

A significant correlation was observed between the distance travelled by the rats in the OF test and their ethanol intake scores ($r = .7$, $p < .001$; see Figure 13).

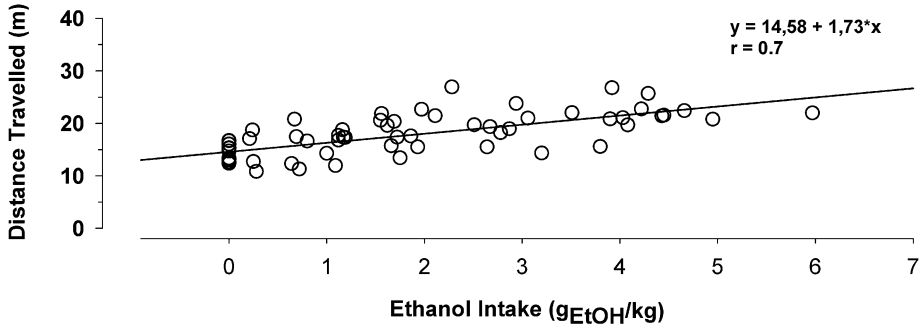

Figure 13. Association between distance travelled (m) at the Open Field test and ethanol intake scores (g_{EtOH}/Kg) achieved by Wistar rats. Independent Pearson correlation coefficients indicated that greater ethanol intake was significantly associated with greater distance travelled.

Likewise, the correlation between BELs and ethanol intake on the day of blood sampling was significant ($r = .65$, $p < .05$; see Figure 14).

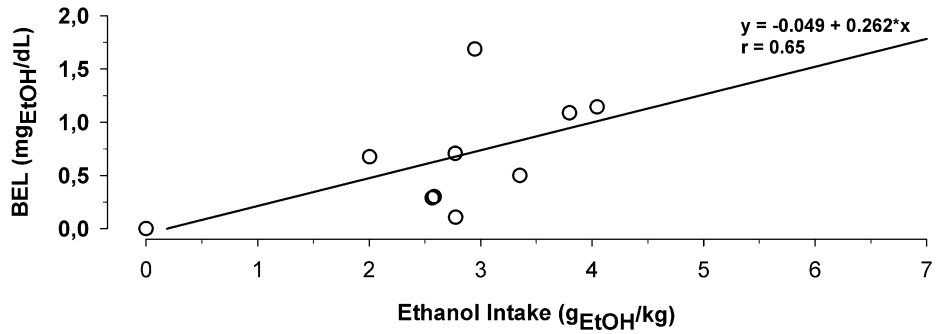

Figure 14. Association between blood ethanol level (mg_{EtOH}/dl, BEL) measured on the day of sampling and ethanol intake scores (g_{EtOH}/Kg) achieved by Wistar rats. Independent Pearson correlation coefficients indicated that greater ethanol intake was significantly associated with higher BELs.

Experiment 2 – From BE to BD model: intake despite aversive consequences

One rat was identified as non-responder to the BE protocol (i.e., exhibited a complete avoidance of the DPPs or of the ethanol solution) and thus was excluded from the experiment. The ANOVA for gEtOH/kg during sessions 11 to 15 yielded a significant interaction between Group (Control group receiving plain 10% ethanol or Quinine Adulterated group) and Session ($F_{4,68} = 5.6$, $p < .01$, $\eta^2p = .25$). As confirmed by the post-hoc tests, the adulteration with .01, .03 or .1 g/L quinine failed to decrease ethanol intake. Only the addition of 0.3g/L of quinine led to a significant decrease in ethanol intake. A graphical representation of these results could be found in Figure 15.

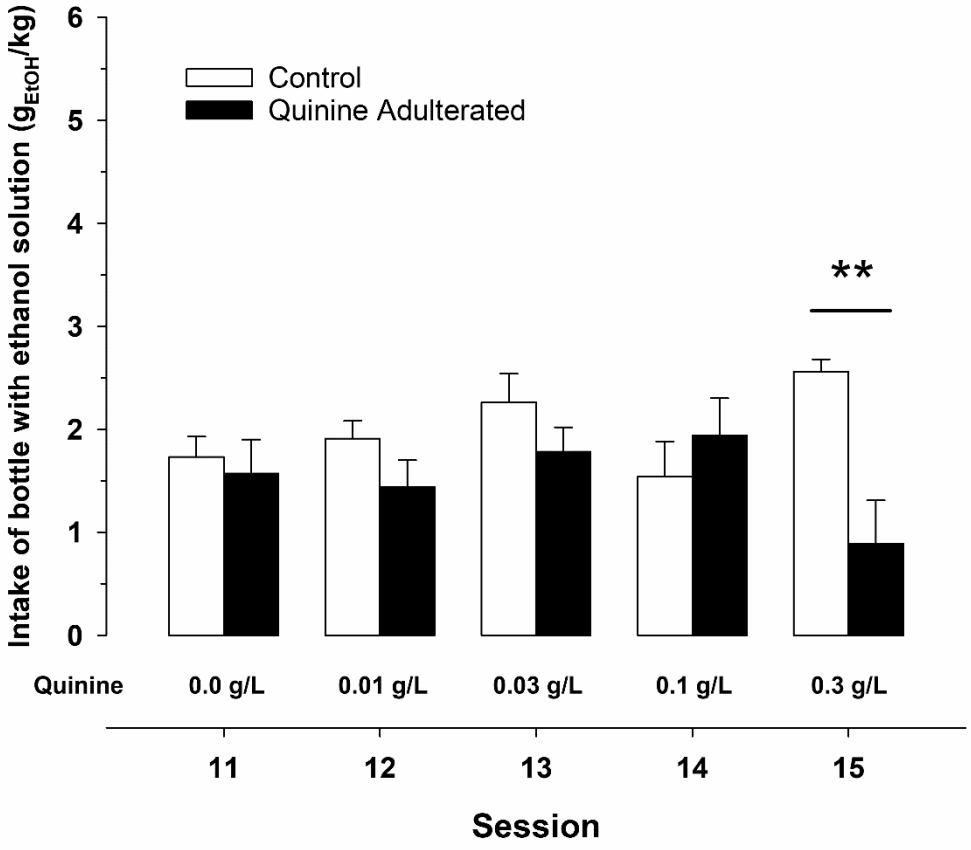

Figure 15. Effects of the adulteration of the ethanol solution (10% w/w) with increasing quinine concentrations (0.01 – 0.3 g/L, from session 11 to 15 respectively). Control rats were given exposure to unaltered 10% w/w ethanol. Each bar and vertical line represent the mean ±SEM of the values obtained in 9-10 animals per group. Statistically significant differences in ethanol intake scores between Control and Quinine Adulterated group on session 15 (**p<0.01).

Experiment 3 – From BE to BD model: assessing predictive validity

Twelve rats were identified as non-responders to the BE protocol (i.e., exhibited a complete avoidance of the DPPs or of the ethanol solution) and thus were excluded from the experiment. The ANOVA for gEtOH/kg during sessions 11, 12 and 13 yielded a significant interaction between Naltrexone dose and Session ($F_{4,44} = 3.69$, $p < .01$, $\eta^2p = .25$) (see Figure 16). Given our a *priori* hypothesis, a planned comparison was conducted, which indicated that ethanol drinking was lower in rats treated with 10 mg/kg naltrexone, when compared to those which received 1 mg/kg or vehicle on session 12. There were no significant differences between the groups at sessions 11 or 13 (i.e., non-injection sessions).

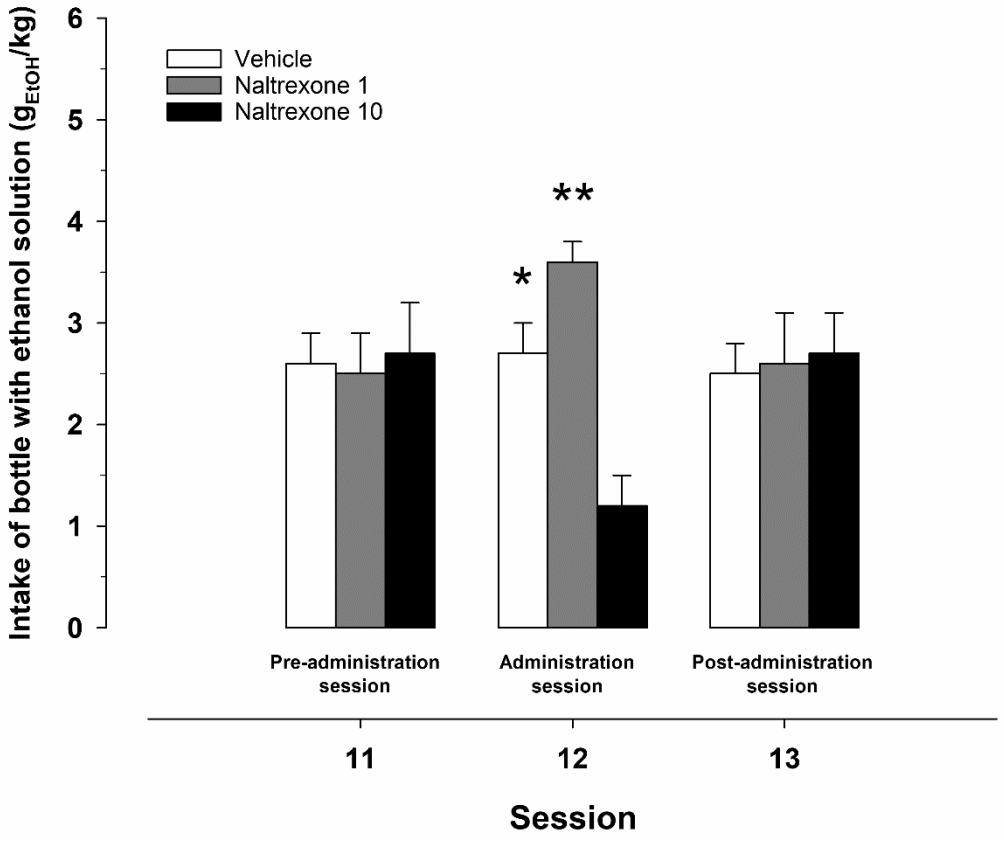

Figure 16. Effects of the subcutaneous administration of naltrexone (1 or 10 mg/kg, grey and black bars respectively) on ethanol intake (g_{EtOH}/Kg) during session 12 (administration session), in comparison to sessions 11 and 13 (pre- and post-administration sessions, respectively). Each bar and vertical line represent the mean ±SEM of the values obtained in 6-12 animals per group. Statistically significant differences between ethanol intake scores obtained in vehicle and 1 mg/kg naltrexone-treated animals compared to 10 mg/kg naltrexone-treated animals: *$p < 0.05$; **$p<0.01$.

Experiment 4 – From BE to BD model: modulation of S1-R

In this experiment, a total of 21 rats were identified as non-responders to the BE protocol (i.e., exhibited a complete avoidance of the DPPs or of the ethanol solution) and thus were excluded from the experiment.

Regarding the procedure that was carried out to assess the effectiveness of acute administration of BD-1063 on the self-administration of 10% (w/w) ethanol solution (Figure 17, panel A), the ANOVA conducted during sessions 11 – 13 yielded a significant main effect of Session ($F_{2,44} = 4.41$, $p < .05$, $\eta^2p = .17$), with greater intake for session 13 compared to session 11, as confirmed by the post-hoc tests. No effect of the treatment was found at the doses administered. There was not a significant effect of the treatments with S1RA or PRE-084 (Figure 17, panel B and C) on ethanol intake either (at 6% w/w for the experiment with the antagonist or 10% w/w with the agonist) ($F_{2,54} = .45$, $p > .05$; and $F_{2,44} = 2.74$, $p > .05$ respectively).

Daily preventive treatment with 64mg of S1RA was also found to be ineffective in ethanol self-administration in this model, as can be seen in Figure 18. ANOVAs for sessions 5 to 7 and 8 to 14 reveal a significant effect of Session ($F_{2,18} = 4.86$, $p < .05$, $\eta^2p = .35$; and ($F_{6,54} = 11.48$, $p < .001$, $\eta^2p = .56$, respectively), with a higher ethanol intake for the latter sessions.

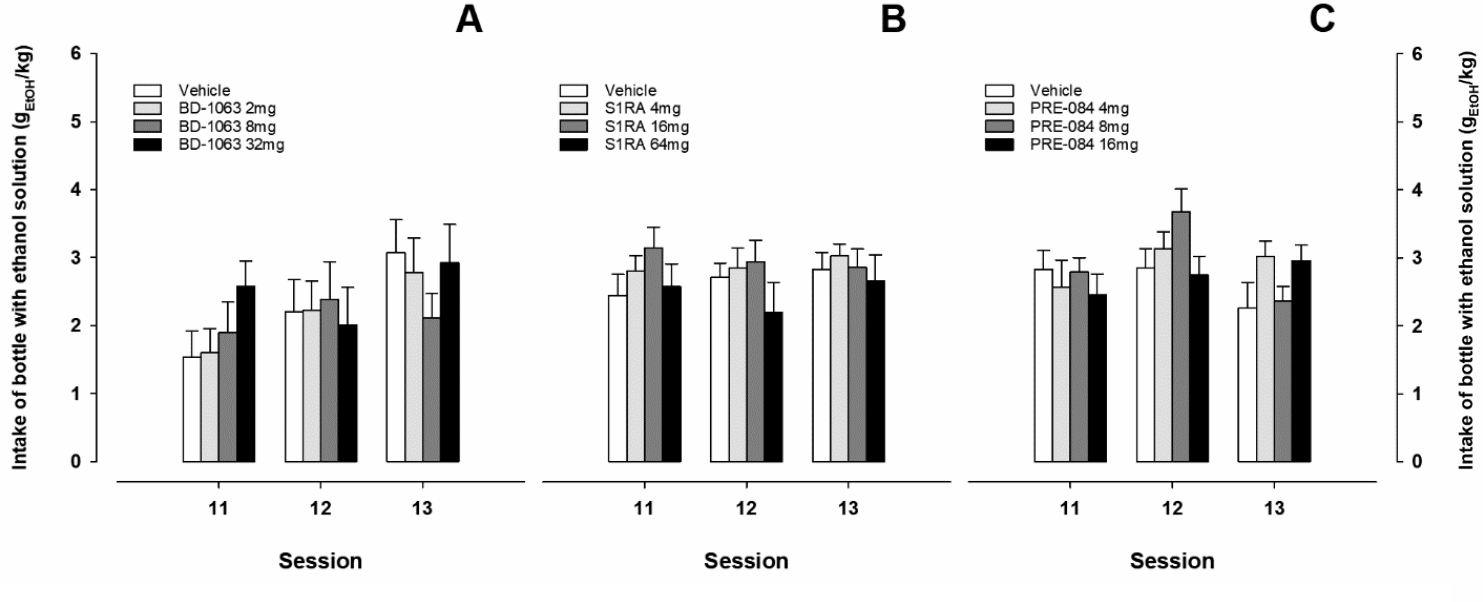

Figure 17. Effects of the subcutaneous acute administration of the S1-R antagonists BD-1063 (0, 2, 8 or 32 mg) or S1RA (0, 4, 16 or 64 mg); or the S1-R agonist PRE-084 (0, 4, 8 or 16 mg) on ethanol intake (g_{EtOH}/Kg) during session 12 (administration session), in comparison to sessions 11 and 13 (pre- and post-administration sessions respectively). Each bar and vertical line represent the mean ±SEM of the values obtained in 6 – 9 animals per group.

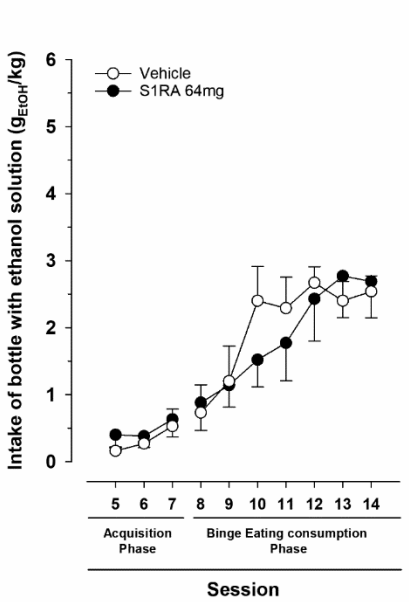

Figure 18. Effects of the subcutaneous daily administration of the S1-R antagonist S1RA (64 mg; n = 7) or vehicle (n = 4) on ethanol intake (gEtOH/Kg) of a 6% (w/w) ethanol solution across sessions. Each point and vertical line represent the mean SEM.

Experiment 5 – BD in adolescence: effect of S1-R antagonism.

In Experiment 5A, all intake rates (g/kg_{EtOH}, $\%_{EtOH}$ and overall fluid intake) were similar for male and female during the habituation session ($p > 0.05$, see Supplementary table 1). The ANOVA for g/kg ingested during the binge sessions (Figure 19) yielded significant main effects of Dose ($F_{3,70} = 34.08$, $p < .001$, $\eta^2p = .59$), Session ($F_{5,350} = 58.17$, $p < .001$, $\eta^2p = .45$) and Sex ($F1,70 = 28.2$, $p < .001$, $\eta^2p = .29$), and significant two-way interactions between Session and Sex ($F_{5,350} = 4.59$, $p < .001$, $\eta^2p = .06$), and between Session and Dose ($F_{15,350} = 3.13$, $p < .001$, $\eta^2p = .13$). The interaction Session x Sex x Dose was also significant ($F_{15,350} = 3.38$, $p < .001$, $\eta^2p = .13$). In each binge session, male and female rats given 64 mg/kg S1RA drank significantly less than their sex-matched vehicle counterparts. Male rats given 4 or 16 mg/kg S1RA drank significantly less than those given 0 mg/kg in session 3 or in session 1 and 2 respectively, whereas female rats given 4 or 16 mg/kg drank significantly less than females given 0 mg/kg in session 2 – 5 or in sessions 2 – 6 respectively.

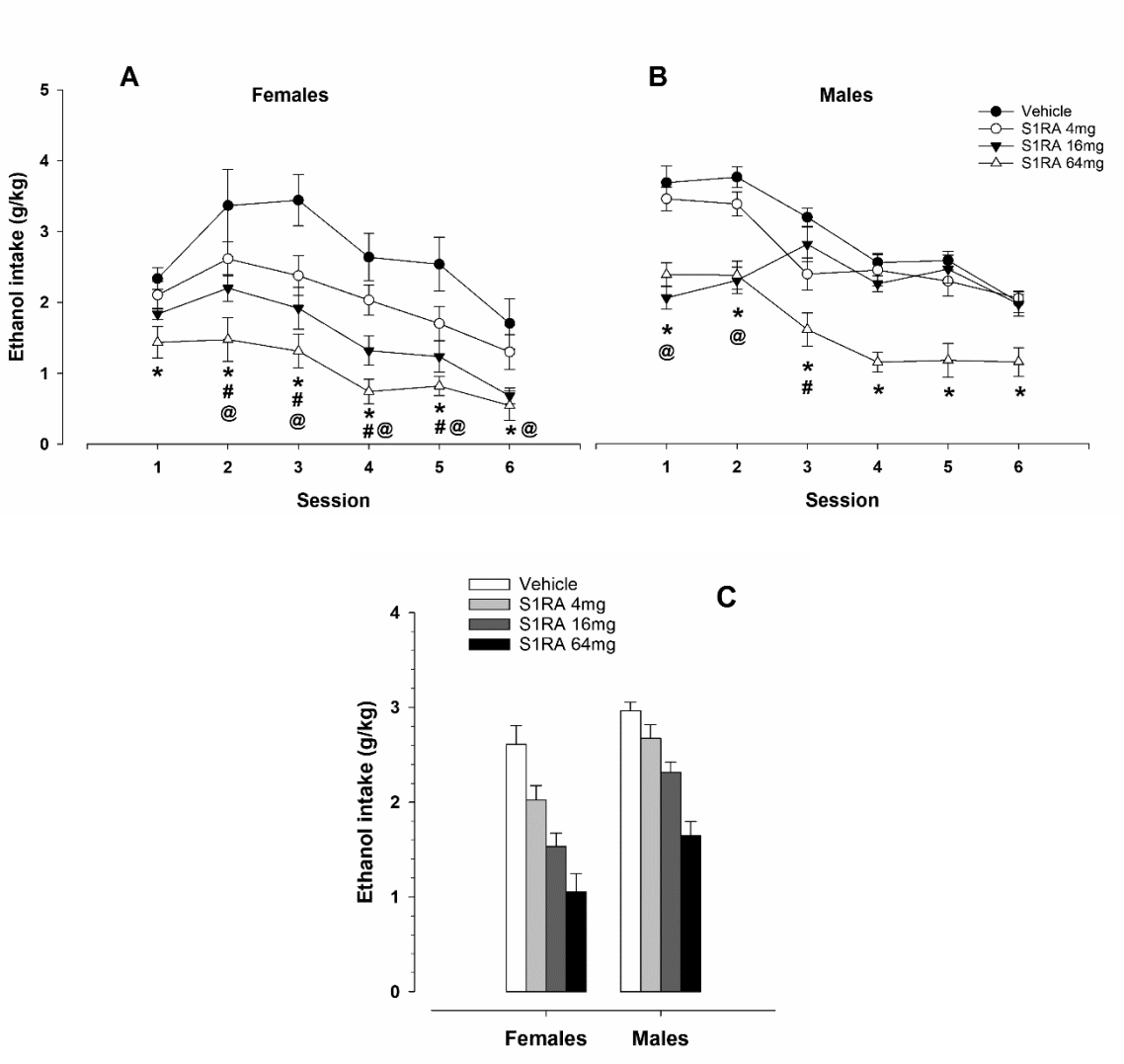

Figure 19: Panels A-B. Ethanol intake (g/kg) in adolescent Wistar rats as a function of sex (females, males), binge intake session (i.e., Sessions 1 – 6) and treatment with S1RA (4, 16, 64 mg/kg or vehicle). **Panel C**. Same data as in A-B but collapsed across sessions. In Experiment 5A the ANOVA yielded a significant Treatment x Sex x Session interaction. The female rats given 64 mg/kg or 4 mg/kg S1RA drank significantly less than the 0 mg/kg group on days 1 – 6 or 2 – 5 respectively. These differences are indicated by the * and # signs. Among females, the 0 mg/kg group and the 16 mg/kg S1RA group differed significantly on days 2 – 6, as indicated by the @ sign. The male rats given 64 mg/kg or 16 mg/kg S1RA drank significantly less than the 0 mg/kg group on days 1 – 6 or 1 – 2 respectively. These differences are indicated by the * and @ signs. Males treated 0 mg/kg or 4 mg/kg S1RA differed significantly only on day 3, as indicated by the # sign. The data are expressed as mean ± SEM of the values obtained in 10 rats per group.

Regarding Experiment 5B, males and females exhibited similar ethanol (g/kg) or liquid intake at the habituation (Supplementary table 1), yet females exhibited significantly higher percent ethanol intake than males did ($t = 2.58$, $p<0.001$; 14.66 vs 6.53). The ANOVA for g/kg ingested during the binge sessions revealed significant main effects of Dose and Session ($F_{3,62} = 8.88$, $p < .001$, $\eta^2p = .30$ and $F_{5,310} = 32.63$, $p < .001$,

η^2p = .34) (Figure 20). As confirmed by the *post-hoc* tests, ethanol drinking (g/kg) was significantly higher in binge days 1 and 2 than in day 3 and significantly greater in binge days 1, 2 or 3 compared to days 4 to 6. Across days the rats treated with 32 mg/kg BD-1063, both males and females, drank significantly less than the other groups.

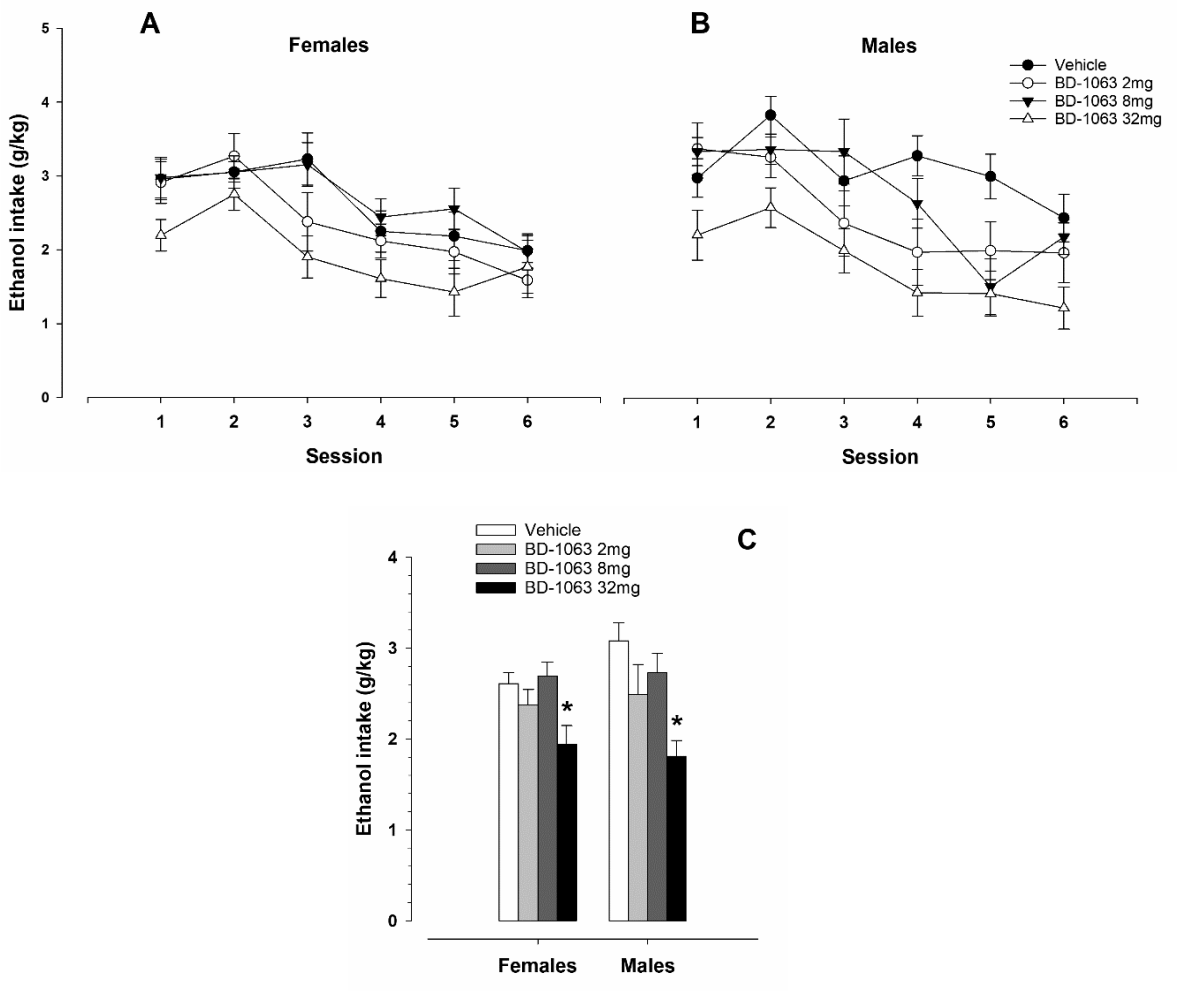

Figure 20: Panels A-B. Ethanol intake (g/kg) in adolescent Wistar rats as a function of sex (females, males), binge intake session (i.e., Sessions 1 – 6) and treatment with BD-1063 (2, 8, 32 mg/kg or vehicle). **Panel C**. Same data as in A-B but collapsed across sessions. In Experiment 5B the ANOVA indicated a main effect of treatment: the rats treated with 32 mg/kg BD-1063, both males and females, drank significantly less than the other groups, an effect indicated by the * sign in panel C. The data are expressed as mean ± SEM of the values obtained in 7 – 10 rats per group.

Post-test ethanol drinking scores (see figure 21) were significantly lower in males or female rats treated with 16 or 64 mg/kg S1RA and in females treated with 4 mg/kg, than in vehicle-treated controls (significant Sex x treatment interaction, $F_{3,70} = 3.50$, $p < .05$, $\eta^2p = .13$ and $F_{3,69} = 4.49$, $p < .01$, $\eta^2p = .16$, for g/kg and $\%_{EtOH}$ respectively). Liquid ingestion (Supplementary table 1) was lower in rats treated with either dose of

S1RA than in vehicle-treated rats (F3,69 = 7.80, p < .001, η^2p = .85). Regarding the effect of BD-1063, the variables measured at the post-test (Figure 21 and supplementary table 1) were not significantly affected by BD-1063 nor by sex. Guided by *a priori* hypothesis, we conducted a planned comparison on g/kg scores between the 0 mg/kg and the 32 mg/kg group, which revealed significantly less ethanol consumed in the 32 vs. the 0 mg/kg group ($F_{1,62}$ = 4.05, p < .005). The ANOVAs for NOR scores (distance travelled at habituation or Di scores at test, Supplementary table 1) did not reveal significant main effects or significant interactions.

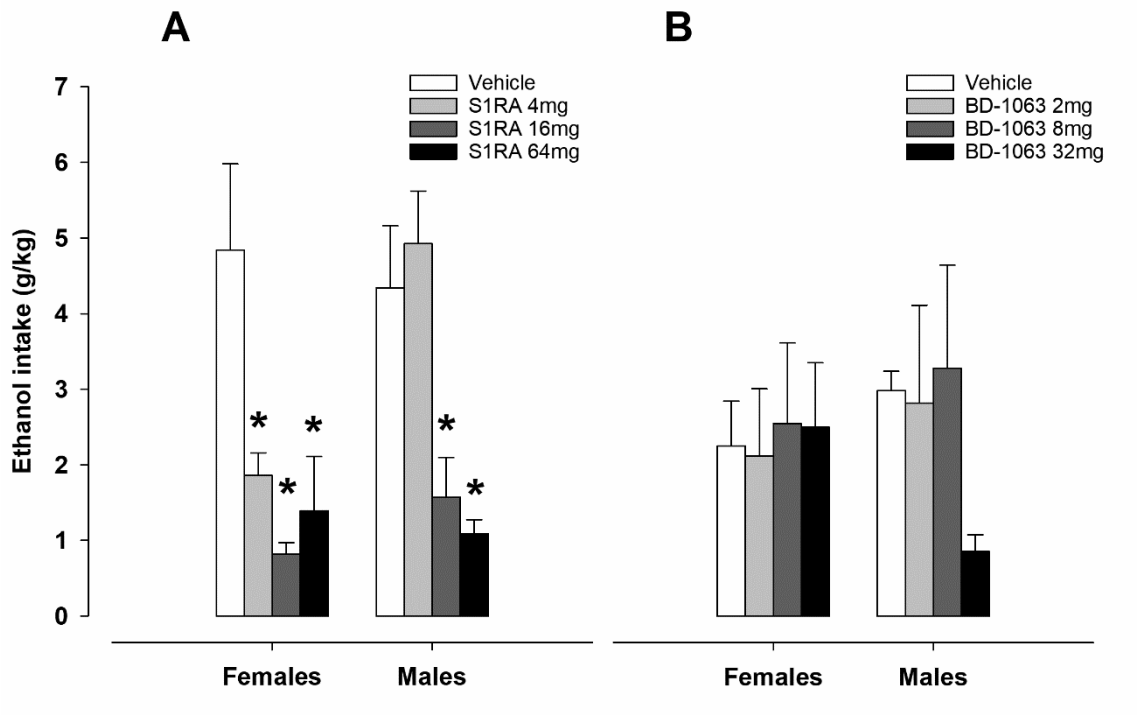

Figure 21: Ethanol intake (g/kg) in adolescent Wistar rats as a function of sex (females, males), in 2-BC post-test session. **Panel A**. Male rats treated with 16 or 64 mg and female treated with 4 – 64 mg of S1RA drank significantly less ethanol solution than their vehicle-treated sex-matched control. **Panel B**. The ANOVA for the effect of BD-1063 on ethanol intake in post-test session did not yield a significant effect. Guided by a priori hypothesis, a planned comparison on ethanol intake between the 0 mg/kg and the 32 mg/kg group was conducted, which revealed significantly less ethanol consumed in the 32 vs. the 0 mg/kg group. The data are expressed as mean ± SEM of the values obtained in 7 – 10 rats per group.

Discussion

1. From Binge Eating to Binge Drinking model

In this Doctoral Thesis, a new preclinical model of voluntary ethanol consumption in adult male rats is presented and validated, at least partially. To the best of our knowledge, the present preclinical model is the first one that resembles the interaction between the compulsive addictive behaviors of BE and BD, and it induces remarkable ethanol self-administration levels (that is, around 5g/kg/90min) and ethanol preference scores neared 80 – 90%, much greater than dose reported by other preclinical models. For instance, rats exposed to intermittent access to 20% ethanol in a two-bottle-choice procedure achieved 9-10 g/kg, yet they did that in a 24h span (Carnicella et al., 2014), whereas those tested in the Drinking-in-the-dark-multiple-scheduled-access rarely exceed 5-6.5 g/kg/day (Colombo et al., 2014).

The presented results indicate that the experience of BE immediately before the availability of ethanol heightens voluntary ethanol self-administration, particularly with the 6% and 10% (w/w) ethanol concentrations. When using the level of preference for the "experimental" bottle (i.e., a bottle filled with 0, 6, 10 or 14% ethanol) as a point of reference across groups, it seems that exposure to the BE condition was associated with a notable preference for the alcohol solution, over 0.85 in most groups. An exception to the latter pattern was when the rats were given the 14% ethanol concentration, which was associated with ethanol intake and preference similar to or lower than those observed in control counterparts. This might relate to rodents' well-known reluctance to ingest highly concentrated ethanol solutions, which may entail burning sensation or gastric irritation (Kiefer & Dopp, 1989).

When two bottles of water, or water and a 14% ethanol solution were available, water intake was greater in those rats that indulged in BE. In other words, when no ethanol solution was available, BE exerted a facilitatory effect on water intake, which suggests that BE does have a facilitatory effect upon general fluid intake (see Supplementary figure 1, top panel). Nevertheless, it is important to highlight that, when ethanol was available in two-bottle choice against water, BE yielded a highly selective facilitatory effect on ethanol consumption. Specifically, when the rats that underwent BE were offered a bottle of 2, 6 or 10% ethanol (and a bottle of water) there was a notable increase in overall fluid intake, which was: (a) much greater than that observed after 6 DPP and (b) specifically driven by the drinking from the ethanol bottle (note that drinking from the water bottle was negligible in these groups; see Supplementary figure 1 – middle panels).

It is notable that some characteristics of the present intake model resemble those of the scheduled-induced polydipsia (SIP) model (Falk & Tang, 1988). The latter preparation leads to relatively high levels of ethanol intake by exposing food-deprived rats to several sessions of pellet delivery with a time-fixed interval. There are, however, methodological differences that mean it is unlikely that the findings of the present study adhere to SIP. Specifically, it has been found that for SIP to take place an interval of 40 to 60 seconds is needed between pellet delivery. Moreover, SIP involves the presentation of several pellets across a rather lengthy session (usually within 1 hour), and the longer the session the greater the SIP (Falk & Tang, 1988). In sharp contrast, in the present model all pellets are presented at the same time. Also, in the SIP model, most of the liquid intake occurs in the training box during the interval between pellet delivery, yet the amount of fluid the rats drink in their home-cages is negligible (Falk, 1966). In the present study, the rats are exposed to the ethanol or water bottle in their home-cage, hence fluid intake and pellet exposure occur in quite different and distinct contexts.

The self-administration of ethanol of this model is subject to several possible interpretations. The comorbidity between AUD and other disorders, including the addiction to other substances, is well-known. In that sense, clinical data shows connections between BD and BE (Birch et al., 2007; Munn-Chernoff et al., 2021), and similarities between both BE and BD can be observed. Modeling BE implies the ingestion of high amounts of palatable foods (lipids or sugars) in short periods of time (Cottone et al., 2012; Heyne et al., 2009), just as BD implies high consumption of ethanol in a brief period of time. In addition, although the BE condition was not spontaneous in our model (because the animals were slightly food deprived), the consumption pattern (amount of food per minute) was much more pronounced than that found in other models of BE (11.66 kcal/3min vs. 2 kcal/3min38), reaching even similar levels of caloric consumption with a lower consumption time (11.66kcal/3min vs 12kcal/30min) (Curtis et al., 2019).

Another factor that could explain the patterns found relates to the high sucrose content of the DPP (59.1% carbohydrate). It has been shown that, in rats, a previous alcohol (or sugar) dependence, induced by a binge procedure, facilitates subsequent sugar (or alcohol) consumption (Avena et al., 2004). It is important to note that, in mice, this ethanol-food interaction has also been reported with fat bingeing (Blanco-Gandía et al., 2018; Blanco-Gandía, Ledesma, et al., 2017), highlighting the key role of BE of high hedonic value foods in drug abuse (Avena et al., 2008; Blanco-Gandía, Cantacorps, et al.,

2017; Blanco-Gandía, Ledesma, et al., 2017; Brutman et al., 2020; Puhl et al., 2011). Furthermore, the sweet neural pathway seems to be essential to voluntary ethanol consumption, as long as rats sense such taste in ethanol solutions (Lemon et al., 2004) and its suppression produces a reduction of ethanol intake (Blednov et al., 2008). This relationship between the taste of ethanol and sweetness may play a crucial role in the ethanol intake in this model.

It is conspicuous that previous studies which employed stimulation with 32% sucrose failed to induce subsequent self-administration of 2% ethanol (Manzo et al., 2015). It is noteworthy that, in that previous study, sucrose consumption was actually higher (around 2.4 g) than that observed in our experiment (i.e., around 1.91 g). This has several implications. One relates to a sequential effect of sucrose on alcohol consumption, which to our knowledge has not been investigated before. Previous research has analysed the mutual interaction of alcohol and sucrose but by using an administration pattern that provides them simultaneously or separated by a significant delay (Avena et al., 2004; Dorofeikova et al., 2017; Ji et al., 2008; Vendruscolo et al., 2010), which significantly differs from the sequential pattern of exposure employed in the present work.

In contrast to the results of Blanco-Gandía et al (2018), there are studies that have reported an attenuation of ethanol intake after binge-like consumption of a high-fat diet (Sirohi et al., 2017). However, aside from the key fact that one study employs rat and the other mice, the presentation time of the diet is 2 vs. 24 hours, which highlight the importance of the presentation method in the influence of BE on the subsequent consumption of ethanol.

Moreover, as mentioned before, BE activates similar brain regions as drug of abuse (Blum et al., 2017; Volkow et al., 2011) and is found to be sensitive to manipulations of the opioid (Karkhanis et al., 2017) and DA system (Avena et al., 2008; N. T. Bello & Hajnal, 2010; Berner et al., 2011). It is possible that BE stimulates, and likely primes, the brain reward pathway and, due to such priming, promotes the subsequent consumption of ethanol. Future research should include neurobiological measurement with the purpose of corroborating this hypothesis.

An important limitation of the present study is the lack of female representation. Future studies should include both sexes to assess potential sex-related patterns in the phenomena under analysis, as reported in other models (Hilderbrand & Lasek, 2018).

2. Validity of the "From Binge Eating to Binge Drinking model"

Currently, progress in research into alcoholism (or any psychiatric disorder) requires the use of animal models that resemble, at least partially, human conditions, that is, that demonstrate validity in different aspects.

Face validity refers to whether there are equivalences in terms of behavior and symptoms between the preclinical model and humans (Ciccocioppo, 2012). In the model presented throughout this Doctoral Thesis the ethanol consumption occurs quickly, voluntarily and in large quantities, reaching up to 6.3 g_{EtOH}/Kg, features that also meet the BE episodes during the BE consumption phase. Furthermore, rats in this model exhibit a positive correlation between ethanol intake and BELs or distance travelled, the latter related to the well-known ethanol stimulant effect, thus displaying signs resembling human BD. It could be argued that a weak point in the face validity of this model as a model of BD is the lack of day off between session (i.e., leave one or several days between sessions of consumption), since BD in human mainly occurs intermittently across days (Courtney & Polich, 2009). Although population studies also show daily consumption patterns that could fit the consumption in the present model (Hedden et al., 2015; Observatorio Español de las Drogas y las Adicciones, 2021), future research should test the effect of days off between days of consumption in the present model in order to increase its validity.

The resistance to quinine adulteration of ethanol solution (at concentrations of 0.01 – 0.1 g/L) in a model of voluntary ethanol consumption is assumed to be a proof of compulsive or habitual consumption, since it occurs despite aversive consequences (Hopf & Lesscher, 2014; Radke et al., 2020). The consumption of bad-tasting alcoholic beverages occurs in people once alcohol dependence is established (Leon et al., 2007), a behavior also shown by the rats in the "From BE to BD" model for both the ethanol solution and the DPPs when adulterated with quinine.

As predicted, the ethanol self-administration of the rats exposed to BE and 10% ethanol solution was significantly reduced after the acute administration of naltrexone. A large body of evidence supports the mediating role of opioid receptors in ethanol intake, and the antagonism of this system is a common therapeutic target in AUD treatment (Guardia Serecigni, 2015; Sudakin, 2016). This result demonstrates the predictive validity of the model, insofar as the model is sensitive to an approved drug for AUD in humans.

Likewise, it also represents a test of the construct validity of the model since it shows that consumption in it is mediated, as in the case of consumption in humans, by the opioid system, at least partially. However, future research that seeks to continue evaluating the construct validity of this model should confirm the involvement of other circuits or brain systems already linked to the acquisition and maintenance of ethanol consumption as DAergic or GABAergic systems (Bell et al., 2017). Furthermore, to improve predictive validity, other pharmacotherapies should be evaluated in the present model.

To summarize, this novel model of ethanol consumption induced by a BE experience involves a new approach significantly different approach from other ethanol self-administration methods like operant models that require lever pressing, or chronic or episodic access, or the drinking in the dark procedure (Bell et al., 2017; Jeanblanc et al., 2019). The BE-BD association triggers an important amount of ethanol consumption in a relatively brief period, which can seldom be observed in other self-administration paradigms, and thus the model seems to better fit the canonical definition of binge drinking. Furthermore, since the model does not require extensive ethanol pre-exposure or habituation, the model allows the investigation of all the stages of the ethanol addiction and the addiction of a new experimental tool for drug discovery in the AUD field.

3. Effect of S1-R modulation on ethanol consumption

As mentioned before, targeting S1-R is a promising pharmacological strategy for treating drug abuse in general and particularly AUD (Quadir et al., 2019). Several studies have shown the effectiveness of the treatment with S1-R antagonists (mostly throughout the administration of BD-1063). For instance, the latter has been reported to reduce ethanol self-administration in ethanol-naïve sP rats (namely, rats selectively bred for ethanol intake) and ethanol-dependent Wistar rat during withdrawal from operant ethanol self-administration. However, the treatment with BD-1063 (at doses 4.4 – 11 mg/kg) treatment was ineffective in reducing ethanol intake in non-dependent Wistar rats (Sabino, Cottone, Zhao, Iyer, et al., 2009).

Regarding the effectiveness of the treatment with BD-1063 in the "From BE to BD" model, in terms of ethanol consumption, rats did not respond to the treatment regardless of the doses tested. Taking these results into account, the subsequent test to assess the effect of S1-R antagonism on ethanol consumption in the model was carried out by reducing the concentration of the offered ethanol solution (from 10% to 6% w/w) to avoid a possible "ceiling effect" produced by it. Despite this methodological change, both acute and preventive treatment with S1RA did not have a significant impact on ethanol self-administration in the "From BE to BD" model. These results, which are consistent with previous studies in non-dependent Wistar rats (Sabino, Cottone, Zhao, Iyer, et al., 2009), demonstrate the ineffectiveness of S1-R antagonism in our model, a lack of effect that does not seem to be drug dependent since neither of the two antagonists tested produced changes in consumption.

This lack of effectiveness of the S1-R antagonism on ethanol self-administration in our BE-BD model was particularly unexpected considering that, in addition to the known actions on ethanol self-administration, the treatment with a novel selective S1-R antagonist has been reported to dose-dependently block episodes of BE in female rats exposed to a stress-related BE model (F. del Bello et al., 2020). However, the intake of DPPs in our model was also not affected by either treatment.

Correspondingly, the acute treatment with the S1-R selective agonist PRE-084 was also found to be ineffective with respect to modifying the ethanol intake in the BE-BD model, which is consistent with other attempts to promote ethanol intake through the administration of PRE-084 (Salguero et al., 2022). Nevertheless, repeated treatment with

the S1-R agonist DTG has been shown to increase ethanol self-administration in an operant paradigm in sP rats (Sabino et al., 2011; Valenza et al., 2020).

S1-R agonists has been shown to promote the rewarding effect of ethanol (Maurice et al., 2003; Sabino et al., 2011) and restore the conditioned reinforcing effect in CPP situation (Bhutada et al., 2012). It is possible that this promoting effect of the S1-R agonism over the rewarding properties of ethanol also affect the BE-BD model. However, it is not an effect that we have been able to verify because we carried out the administration in the consumption plateau during the BE consumption phase. Further research should assess the effect of PRE-084 in the early stages of the model, that is, when ethanol intake has not peaked, in order to elucidate its actions.

Contrastingly, the results yielded by the studies in adolescent rats showed an effect of S1-R antagonism. More precisely, the administration of S1RA or BD-1063 blocked ethanol binge drinking in both male and female adolescent Wistar rats. Adolescents given 64 mg/kg S1RA exhibited, relative to vehicle-treated counterparts, an overall two-fold reduction in ethanol drinking. The influence of both treatments largely surpassed the threshold of a large effect (i.e., $\eta^2p \geq 0.14$). To our knowledge, this is the first report of S1-R modulating ethanol consumption at adolescence, and the first to show interaction between the S1-R system and ethanol intake in females.

In Experiment 5, S1-R antagonism diminished ethanol intake of females in a dose-dependent manner, whereas males displayed a suppressing effect mainly after the highest S1RA dose. Studies have shown that ethanol drinking of female rats is more affected by stress than that of male rats (Varlinskaya et al., 2015; Wille-Bille et al., 2017), suggesting that females are more like to drink ethanol to reduce anxiety. Our adolescent binge-drinking model involves brief yet significant social isolation and a mild liquid deprivation that can serve as stressors. It is possible that low doses of S1RA are selectively effective at reducing ethanol drinking driven by the drug's anxiolytic effects. This is just a hypothesis, yet it is intriguing that the selective S1-R antagonist NE-100 dose dependently blocks ethanol intake in the stress-sensitive and anxiety-prone Sardinian alcohol preferring rats (Sabino, Cottone, Zhao, Steardo, et al., 2009). Moreover, S1-R binds compounds that effectively for treat anxiety during adolescence (Cheer & Figgitt, 2002) and S1-R activation mediates stress effects in animal models of gastrointestinal tract disorders seen in anxiety (Gue et al., 1992).

S1RA had a lingering effect, reducing absolute ethanol drinking vs. vehicle-treated controls at the two-bottle post-test, long after its clearance. The effect was seen across S1RA doses for females, and there was some evidence of this effect after 32 mg/kg BD-1063. S1RA achieves the maximum plasma concentration shortly after its administration to rodents and is quickly metabolized, given that it has a short half-life. After its administration, plasma levels are also undetectable after 6 h and its metabolites are inactive and it does not accumulate in tissues, including the brain (Gris et al., 2016). Thus, the persistent effect of S1RA suggests it alters plastic effects associated with the chronic ethanol exposure. The pattern resembles findings in which naloxone prevented binge exposure and the promoting effect of such exposure upon later drinking (Salguero et al., 2020). It also resembles the findings of studies in which S1-R antagonists blocked cocaine-induced behavioral sensitization (Ujike et al., 1996).

Whether the effect found was reversed by S1-R agonism was not assessed in this experiment. Whether the treatment affected voluntary ingestion of water was not assessed either. However, the latter is unlikely since previous report indicated that the treatment with S1-R antagonists altered ethanol ingestion without significantly altering water or overall liquid ingestion (Sabino, Cottone, Zhao, Iyer, et al., 2009). Furthermore, BD-like ethanol intake in adolescence did not yield ethanol-induced memory impairments measured in the NOR test, thus it was not possible to assess the effectiveness of S1-R antagonism on it. Nevertheless, these limitations should be addressed in future investigations.

Taking all of this into consideration, it must be highlighted that all the procedures for establishing a rodent model in ethanol research have strengths and weaknesses, but choosing models that show higher validity is important for properly mimicking human conditions, because this allows results to be extrapolated with lower probability of failure. Moreover, the discrepant results found throughout the present Doctoral Thesis regarding the effect of S1-R modulation on ethanol intake point out the importance of paying attention to the particular experimental method of each experiment, since variables inherent to the animal model such as the age, sex, species, genetic background, as well as those extraneous to them, like diet composition, exposure time, exposure duration and route of administration, have a strong influence of the results obtained.

Conclusions

1. Procedures performed in “From binge eating to binge drinking” (BE-BD) model led to substantial voluntary self-administration of ethanol in adult male Wistar rats. Furthermore, since the model does not require extensive ethanol pre-exposure or habituation, it allows the investigation of all the stages of ethanol addiction.

2. Once the binge eating consumption phase is establish, ethanol and DPP intake in “BE-BD” model has been shown to be quinine-resistant, and rats exhibit signs of intoxication. Therefore, the model exhibit good face and construct validity since the self-administration occurs despite aversive consequences, it is performed quickly and produce behavioural and physiological effects.

3. The ethanol intake in “BE-BD” model was naltrexone-sensitive, hence the model exhibits predictive validity, and the ethanol intake is driven at least in part by the opioid system.

4. The systemic administration of S1-R ligands in the “BE-BD” model did not produce any modulation on the ethanol consumption. Neither the S1-R antagonists S1RA and BD-1063 nor the S1-R agonist PRE-084 exerted any action. Further investigation should be carried out to elucidate the discrepancy between this report and previous ones on the effectiveness on ethanol intake of drugs for this therapeutic target.

5. S1-R antagonism induced by s.c. S1RA and BD-1063 blocked binge drinking in an ethanol self-administration model in adolescent male and female Wistar rats, further reducing free-choice ethanol intake long after its clearance. This is the first evidence of the effectiveness of S1-R antagonism on ethanol consumption in adolescence and in females.

Resumen

Antecedentes

Podemos asumir que el consumo de alcohol juega un papel fundamental en la cultura, religión y relaciones interpersonales de la mayoría de las sociedades modernas, pero este hecho no se trata de una moda actual, si no que presumiblemente se remonta a etapas prehistóricas de la humanidad, puesto que la fermentación alcohólica es un proceso que ocurre naturalmente en la naturaleza.

Los restos arqueológicos más antiguos que evidencian la preparación intencionada de bebidas de contenido alcohólico se han encontrado en una tumba al norte de China y datan del 7000 a.C (Wang et al., 2021). Desde entonces, y prácticamente en cualquier parte del mundo, hay registros arqueológicos que demuestran como el consumo de bebidas alcohólicas por parte del ser humano ha estado presente en todas las etapas de nuestra historia.

Atendiendo a los escritos, obras pictóricas, cerámicas y esculturas que se han encontrado procedentes de las grandes civilizaciones de la Edad Antigua vemos como el consumo y la producción de bebidas alcohólicas (principalmente productos de fermentación de frutas y cereales) fue adquiriendo con el paso del tiempo un papel muy importante en dichas sociedades, dedicándose gran parte de recursos a los mismos. Incluso, en sociedades o periodos en las que las bebidas alcohólicas han sido reguladas o prohibidas, podemos encontrar que se seguían dando formas de consumo de alcohol entre las personas (Gately, 2008).

Teniendo en cuenta estos antecedentes históricos vemos como el consumo de alcohol ha trascendido civilizaciones y culturas hasta nuestros días, hecho que ha motivado a la ciencia para estudiar las bases inherentes al mismo.

En la actualidad, el consumo de alcohol supone un problema a nivel económico, sanitario y social. Se estima que 2300 millones de personas mayores de 15 años son bebedores habituales, alcanzándose un valor de consumo de alcohol puro per cápita de 6,3 litros en 2016 (Hammer et al., 2018), y pudiendo atribuirse un 5,3% de las muertes a nivel mundial al consumo de alcohol. A nivel español, según los últimos datos del Observatorio Español de las Drogas y las Adicciones (2021), el alcohol es la sustancia psicoactiva más consumida, estimándose que 1,3 millones de españoles entre los 15 y 64

años tiene un consumo de riesgo. Estos niveles de consumo están asociados a más de 60 enfermedades tanto agudas como crónicas (Griswold et al., 2018).

Si nos centramos en el consumo de los más jóvenes (aquellos con edades comprendidas entre los 14 y 18 años), prácticamente la mitad de ellos admiten haber tenido un episodio de "binge drinking" o atracón (BD, es decir, consumo de 4 – 5 bebidas alcohólicas en un periodo de tiempo inferior a 2 horas), el cual es una forma de consumo especialmente perniciosa asociada con gran cantidad de consecuencias negativas a nivel físico y social (Adan et al., 2016; Kuntsche et al., 2017; Wicki et al., 2018), así como al desarrollo posterior de un trastorno por uso de alcohol (de sus siglas en inglés, AUD) (Rial et al., 2020).

Diversos estudios muestran como el consumo de alcohol en el humano está condicionado por distintos factores neurobiológicos y contextuales (Stanesby et al., 2019), entre los que encontramos la asociación entre el consumo de alcohol tipo BD y los atracones de comida o "binge eating" (BE). Se ha encontrado que ambos comportamientos comparten características en cuanto a la forma en la que se da el consumo (Dingemans et al., 2017), además de que activan regiones cerebrales similares (Blum et al., 2017; Volkow et al., 2011) por lo que parece adecuado estudiar ambos comportamientos en conjunto y la relación entre ellos.

El alcohol (en adelante etanol), es un clásico ejemplo de droga inespecífica que ejerce sus acciones sobre gran cantidad de moléculas en el organismo y a distintos niveles (Egervari et al., 2021), dando lugar todo ello tanto a los efectos deseados como perniciosos de su consumo.

Gracias a años de investigación, hoy en día se conocen algunas de las dianas moleculares directas a las cuales se une la molécula de etanol dando lugar a algunos de sus efectos. Entre ellas encontramos al alcohol deshidrogenasa, molécula encargada de su metabolismo y degradación (Mackus et al., 2020) y por lo tanto ha sido asociada tanto al AUD como a otras diversas patologías inducidas por el etanol (Crabb et al., 2004). Por otro lado, encontramos que la molécula de etanol también interactúa con algunos receptores de neurotransmisores en el cerebro como los receptores de GABA tipo A (Olsen, 2018), de serotonina tipo 3 (Gibbs & Chakrapani, 2021), de Glicina (Söderpalm et al., 2017), nicotínicos (Miller & Kamens, 2020) o de NMDA (Naassila & Pierrefiche,

2019) entre otros, todos ellos asociados a diversos efectos tanto agudos como crónicos del etanol.

Subiendo en el nivel de complejidad, también se han encontrado determinadas neuronas sensibles a los efectos del consumo de etanol entre los que encontramos neuronas dopaminérgicas (Buck et al., 2021; Dahchour & Ward, 2022), GABAérgicas (Adermark et al., 2014; Roberto et al., 2021; Roberto & Varodayan, 2017), glutamatérgicas (Bell et al., 2016; Buck et al., 2021) y colinérgicas (Clarke & Adermark, 2015; Ma et al., 2022); y sistemas relacionados con el efecto y consecuencias del consumo de etanol como son el sistema opioide (Alongkronrusmee et al., 2016; Chang et al., 2010; Karkhanis et al., 2017), el sistema serotoninérgico (Sari et al., 2011) o el sistema endocannabinoide (Kunos, 2020).

Particular es el caso del receptor sigma-1 (S1-R) ya que ha sido propuesto como una prometedora diana terapéutica para la adicción a sustancias de abuso (Cobos et al., 2008). Concretamente, el S1-R es una chaperona pequeña que ejerce un papel modulador en la neurotransmisión y se encuentra, entre otros sitios, en áreas cerebrales relacionadas con el circuito de recompensa. Pese a que no se ha evidenciado una interacción directa entre la molécula de etanol y dicho receptor, la modulación farmacológica de S1-R ha demostrado influir sobre las propiedades reforzantes del etanol e incluso su consumo en modelos en roedores de laboratorio (Quadir et al., 2019). El amplio abanico de agonistas y antagonistas para este receptor unido a la eficacia de estos en otras patologías (Cobos et al., 2008), alientan el ensayo de la modulación del S1-R en la investigación preclínica para el tratamiento del AUD.

Gran parte de los avances mencionados han sido posibles gracias a los modelos de experimentación animal, los cuales son una herramienta indispensable para la investigación biomédica y farmacológica (Bell et al., 2017). No obstante, los roedores usados en experimentación biomédica demuestran una reluctancia natural para consumir voluntariamente etanol, al menos en cantidades suficientes para producir efectos farmacológicos o toxicológicos (Becker & Lopez, 2016).

Pese a ello, en la actualidad, existen diversas aproximaciones mediante las cuales producir un modelo de consumo en ratón o rata que exprese algunas de las características humanas a la hora del consumo de etanol. Podemos catalogar los distintos modelos en

tres grandes grupos: a) modelos genéticos o de cría selectiva en los que se producen cepas de roedores con una predisposición innata para el consumo de etanol (Crabbe, 2014; Mayfield et al., 2016), b) modelos de administración forzada o pasiva en los que se tiene un gran control sobre la dosis administrada en detrimento de la voluntariedad en el consumo (D'Souza El-Guindy et al., 2010), y c) modelos de consumo voluntario donde el animal adquiere un papel activo en el consumo (Fritz & Boehm, 2016; Jeanblanc et al., 2019).

Se ha encontrado que, al igual que en humanos, las variables como la edad (Spear, 2015) o el sexo (Flores-Bonilla & Richardson, 2019) influyen a la hora del consumo de etanol en roedores. Además, otras sustancias o incluso el estrés (Camarini et al., 2018) pueden promover el posterior consumo de etanol en ratas y ratones.

Otros estudios con animales de experimentación demuestran la relación entre el consumo de azúcar o dietas con alto contenido en grasa y el consumo de etanol (Avena et al., 2004; Blanco-Gandía et al., 2017), abriendo paso a la posibilidad de modelar en roedores la comorbilidad encontrada en humanos entre BE y BD.

Objetivos

La marcada prevalencia en el consumo de alcohol en la sociedad actual y sus consecuencias en la salud (Griswold et al., 2018) ponen en evidencia la necesidad de profundizar en el conocimiento de los mecanismos que subyacen al consumo de alcohol.

En este escenario, son particularmente importantes aquellos modelos animales que demuestren validez a la hora de emular el consumo humano, sin embargo, estos modelos son escasos, especialmente aquellos que modelan comportamientos complejos como pueden ser la relación entre BE y BD.

De igual forma, los tratamientos farmacológicos para el AUD son también escasos y de relativa eficacia, o mostrando eficacia solamente en pacientes altamente motivados. Por lo tanto, se hace necesaria la investigación para el descubrimiento de nuevas estrategias farmacológicas que muestren mayor efectividad. Ante estos antecedentes la presente tesis doctoral se plantea con los siguientes objetivos:

Teniendo en cuenta la falta de modelos animales de ingesta voluntaria de etanol tipo BD que imiten el comportamiento humano, en la presente Tesis Doctoral el **primer objetivo** propuesto es llenar este vacío, al menos parcialmente, proponiendo un nuevo modelo de autoadministración de etanol tipo BD en ratas. El procedimiento se basa en la exposición repetida y breve a una gran cantidad de pellets de precisión azucarados de gran palatabilidad que conducirán a su consumo como en forma de BE, lo que dará lugar a una alta autoadministración voluntaria de etanol de forma BD.

Con el fin de evaluar la validez del modelo, el **segundo objetivo** fue evaluar si la ingesta de etanol ocurría a pesar de tener un sabor aversivo, lo cual indicaría que el consumo se había vuelto habitual o compulsivo (Hopf & Lesscher, 2014; Radke et al., 2020). Para ello, se realizó un experimento de adulteración de la solución de etanol con quinina buscando comprobar si la autoadministración de solución de etanol por parte de las ratas se volvió resistente a la quinina al final del procedimiento.

El **tercer objetivo** consistió en corroborar si el consumo voluntario en el presente modelo esta mediado, al menos en parte, por el sistema opioide y por lo tanto el tratamiento con naltrexona resultaba efectivo para reducir la autoadministración (Guardia Serecigni, 2015). De cumplirse este objetivo de pondría de manifiesto la validez predictiva (es decir, el valor traslacional) del modelo.

Varios estudios muestran la bidireccionalidad sobre el consumo de alcohol de la modulación farmacológica de los S1-R (Quadir et al., 2019), por lo tanto, el **cuarto objetivo** se planteó evaluar si el tratamiento con agentes farmacológicos cuya diana es el receptor S1 tenía un efecto sobre el consumo de etanol en nuestro modelo.

Finalmente, para continuar con la evaluación de la efectividad de la modulación farmacológica de los S1-R, el **quinto objetivo** se propuso comprobar si el antagonismo S1-R era efectivo en la reducción del consumo de etanol en un modelo de consumo en ratas adolescentes.

Material y Métodos

Animales

Para los estudios se utilizaron ratas Wistar obtenidas de Laboratorios Envigo (Barcelona, España), Charles River (Les Oncins, Francia) o criadas en el Instituto de Investigación Mercedes y Martín Ferreyra (INIMEC-CONICET-UNC; Córdoba, Argentina). Las ratas empleadas en los experimentos en edad adulta tenían aproximadamente 70 – 80 días de edad y las empleadas en experimentos en adolescentes tenían aproximadamente 26 – 27 días de edad.

Las ratas se alojaron en jaulas de policarbonato y se mantuvieron bajo un ciclo de luz/oscuridad de 12 h (luces encendidas a las 6:00 a. m.) en una habitación con humedad y temperatura constantes (50 – 60 % y 21 ºC). Las ratas adultas se alojaron individualmente mientras que las adolescentes se alojaron en parejas. Los experimentos con ratas adultas se realizaron durante el tiempo de luz del ciclo día-noche. Con las ratas adolescentes, parte del procedimiento se realizó al comienzo del ciclo de oscuridad. Los procedimientos realizados con animales de experimentación fueron autorizados por las autoridades pertinentes.

Instrumentos y reactivos

Las soluciones de etanol (2, 6, 10, 14 % m/m) se prepararon diluyendo etanol de grado alimenticio al 96 % (v/v) (PanReac AppliChem, Barcelona, España) con agua. Las soluciones se presentaron a ratas en botellas antigoteo de 150 ml (Classic Drinker de Luxe, Zooplus, Munich, Alemania) en su jaula.

Para la inducción del BE, las ratas fueron expuestas a pellets de precisión sin polvo (de sus siglas en inglés DPP; 45 mg unidad, perfil nutricional 59.1% carbohidratos, 18.7% proteínas, 5.6% grasas, 3.6 kcal/g; Bioserve, Femington, USA) en cajas de policarbonato (42.5 x 26.5 x 15 cm) idénticas a su jaula hogar.

Para la adulteración de las soluciones de etanol con quinina se usó quinina monoclorohidrato dihidrato 90% (Sigma-Aldrich, Madrid, España).

El antagonista opioide naltrexona (Sigma-Aldrich, Madrid, españa), los antagonistas para S1-R S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) y BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (ambos proporcionados por Laboratorios Esteve, Barcelona, España), y el agonista para S1-R PRE-084 (2-(4-morpholinoethyl)-1-phenylcyclohexane-1-carboxylate Hydrochloride) (también proporcionado Laboratorios Esteve, Barcelona, España), fueron disueltos en suero fisiológico y administrados de forma subcutánea (s.c.) a un volumen de 5 mL/kg.

La actividad locomotora se evaluó en una prueba de campo abierto (OF) de 3 minutos para evaluar el efecto estimulante motor de la ingesta voluntaria de etanol. El test OF consistió en un cubo de metacrilato negro (60 x 60 x 60). La prueba fue grabada y posteriormente analizada con el software de rastreo de animales ToxTrack (Rodríguez et al., 2018). La memoria de reconocimiento se evaluó a través de la prueba de reconocimiento de objetos novedosos (NOR). Esta consistió en una arena de forma cuadrada (50 x 50 x 50 cm) que fue explorada por ratas en tres fases (habituación, familiarización y prueba), las cuales fueron grabadas. Se midió la distancia recorrida en la fase de habituación y el tiempo dedicado a explorar los objetos en la fase de familiarización y prueba (Salguero et al., 2020).

Procedimientos

Experimento 1 – Modelo "From BE to BD"

Para este experimento se usaron un total de 91 ratas Wistar macho con 70 – 80 días de edad y un peso medio inicial de 280g (± 37) al inicio de los procedimientos. Las ratas se dividieron en distintos grupos experimental de acuerdo al siguiente diseño: 2 (BE [i.e., 72 DPPs] o control eating condition [i.e., 6 DPPs]) x 5 (ethanol concentration: 0%, 2%, 6%, 10% and 14%), con 6 – 13 rata en cada celda del diseño.

Experimentos preliminares mostraban que la ingestión de 72 DPPs en 3 minutos era una tarea factible para las ratas siempre y cuando el consumo se diera en forma de atracón. Típicamente las ratas consumían la mayor parte de los DPP ofrecidos a partir de la cuarta sesión de exposición a los DPP (ver figura 7). Es de destacar que una vez que el

consumo tipo BE se establece, dicho consumo permanece por encima del 80% aunque los DPP se adulteren con una solución aversiva de quinina 2mM.

El procedimiento del modelo "From BE to BD" comenzó con una leve restricción calórica de las ratas hasta alcanzar un 82 – 85% de su peso corporal (Sesiones 1 – 3). La sesión 4, consistió en una sesión de habituación donde las ratas se colocaron en una jaula de policarbonato vacía durante 3 minutos y luego se devolvieron a su jaula de origen, que estaba equipada en ese momento con dos botellas antigoteo (una con solución de etanol y uno lleno de agua, o dos botellas de agua, según el grupo asignado a cada animal). 90 minutos después de eso, las dos botellas fueron reemplazadas por una botella normal de agua. Finalmente, a las ratas se les dieron 6 DPP y comida en sus jaulas. El objetivo de esta sesión fue familiarizar a las ratas con los estímulos que se presentarían durante las sesiones posteriores.

Durante 10 días consecutivos (sesiones 5 – 14), las ratas se expusieron a un episodio de BE simulado o a un episodio de alimentación control (72 o 6 DPP dependiendo de su grupo experimental. Inmediatamente después, las ratas se sometieron a una prueba de elección de dos botellas (90 minutos de duración), en la que se les presentaron, según el grupo, dos botellas de agua o una botella de solución de etanol (2, 6, 10 o 14 % m/m) y una botella de agua.

Entre sesiones, las ratas recibieron agua ad libitum y comida suficiente para mantener el peso corporal establecido. Una representación esquemática del procedimiento se puede encontrar en la Figura 8.

Se seleccionó una cohorte de 64 ratas escogidas aleatoriamente y de todos los grupos para comprobar la actividad locomotora en el test OF después de las sesiones 12 – 14. También se tomaron muestras de sangre de 11 ratas para determinar los niveles de etanol en sangre (BEL).

Bajo las condiciones experimentales anteriormente descritas, las ratas expuestas a una solución de etanol al 10% (m/m) fueron las que alcanzaron mayores valores de consumo por lo que estas fueron las condiciones que se replicaron para los experimentos 2 y 3 diseñados para comprobar la validez del modelo.

Experimento 2 – Modelo "From BE to BD": consumo a pesar de consecuencias aversivas

Uno de los métodos más comúnmente usados para ensayar si el consumo de una solución se da a pesar de un componente aversivo es la adulteración de la mismo con quinina (Hopf & Lesscher, 2014; Radke et al., 2020). En el experimento 2, se replicaron los procedimientos del experimento 1, pero en las sesiones 12 – 15 las ratas se dividieron en dos grupos: un grupo control (n = 10) y un grupo de solución de etanol con quinina (cuya concentración fue aumentando de las sesiones 12 en delante de la siguiente forma: 0.01, 0.03, 0.1 y 0.3 g/L; n = 10). En este experimento todas las ratas fueron expuestas a BE y la duración del test de doble botella fue de 45 minutos.

Experimento 3 – Modelo "From BE to BD": evaluando la validez predictiva

Un modelo demuestra exhibir validez predictiva cuando responde a tratamientos aprobados para el humano para una condición determinada (Ciccocioppo, 2012). En el experimento 3 se comprobó si se producía una reducción en el consumo de etanol por parte de las ratas tras el tratamiento con el antagonista opioide naltrexona, el cual está indicado para el AUD en humanos tanto por la EMA como por la FDA.

De nuevo se replicaron los procedimientos del Experimento 1 con todas las ratas expuestas a BE (n = 35) y con test de doble botella de 45 minutos de duración. En la sesión 12, 30 minutos antes del procedimiento, se administró una dosis de naltrexona (1 o 10 mg/kg) o vehículo.

Experimento 4 – Modelo "From BE to BD": modulación del S1-R

Dada la eficacia de la modulación del sistema S1-R en estudios previos en otros modelos de consumo (Quadir et al., 2019), el Experimento 4 tuvo como objetivo comprobar si el tratamiento con agonistas y antagonistas para S1-R ejercía un efecto sobre el consumo en nuestro modelo.

Para ello 120 ratas Wistar macho fueron expuestas a las mismas condiciones experimentales que en el caso del Experimento 3 (con la salvedad de que en los experimentos en los que se administró S1RA la solución de etanol ofrecida fue del 6%),

pero las drogas se administraron de acuerdo con los siguientes diseños: administración aguda en la sesión 12 de los antagonistas para S1-R BD-1063 (0, 2, 8 o 32 mg/kg) o S1RA (0, 4, 16 o 64 mg/kg); o administración del agonista PRE-084 (0, 4, 8 o 16 mg/kg). Adicionalmente, se ensayó una estrategia de tratamiento diario preventivo con S1RA (0 o 64 mg/kg) administrado 30 minutos antes de los test de doble botella.

Experimento 5 – BD en adolescentes: efecto del antagonismo S1-R

Pese a que la expresión de S1R está condicionada por la edad (Moradpour et al., 2016) y diversos estudios previos demuestran la influencia del sexo sobre el efecto de las drogas de abuso (Flores-Bonilla & Richardson, 2019; Roth et al., 2004), la influencia de los tratamientos farmacológicos sobre el consumo voluntario en modelos preclínicos que contemplen dichas variables aun no ha sido explorada. Con la finalidad de atajar esta problemática el Experimento 5 consistió en evaluar el efecto del tratamiento con antagonistas S1-R en un modelo de consumo voluntario de etanol tipo BE en ratas adolescentes en ambos sexos.

Más concretamente, 169 ratas Wistar (80 en el Experimento 5A y 89 en el Experimento 5B; de las cuales 40 y 39 eran machos, respectivamente) fueron usadas en un paradigma de consumo voluntario descrito por Salguero et al, (2020). Brevemente, las ratas (con 26 – 27 días de edad) fueron evaluadas en un test de doble botella (8% v/v vs. agua) a modo de sesión de habituación. Tras ello se sucedieron 2 semanas de exposición intermitente a una sola botella (8% v/v sesiones 1 y 2 y 10% v/v sesiones 3 a 6) con solución de etanol durante 120 minutos, 3 días por semana. La botella se proveía a las ratas 15 minutos después del inicio del ciclo de noche. El día anterior a cada sesión de exposición a la botella con solución de etanol las ratas recibieron el 50% del agua que usualmente consumían. Finalmente, 72 horas después de la última sesión de consumo, se realizó un nuevo test de doble botella (8% v/v vs. agua).

En los Experimentos 5A y 5B se empleó un diseño factorial de 4 (S1RA: 0, 4, 16 o 64 mg/kg o BD-1063: 0, 2, 8 or 32 mg/kg, respectivamente) x 2 (Sex). Los fármacos fueron administrados 30 minutos antes de cada sesión de BD. Adicionalmente, una cohorte de ratas derivadas del Experimento 5B (6 de cada grupo) pasó por una prueba NOR (ver Figura 9).

Análisis estadísticos

De acuerdo a los experimentos preliminares donde se estableció que las ratas tardaban un total de 3 sesiones en alcanzar un consumo de DPP tipo BE, se decidió realizar los análisis estadísticos de los experimentos en los que se usaba el modelo "From BE to BD" dividiendo las sesiones de consumo en dos fases, la fase de adquisición (sesiones 5 – 7) y la fase de consumo tipo BE (sesiones 8 – 14).

Las variables de consumo para los distintos experimentos fueron analizadas vía RM-ANOVAs, mientras que las correlaciones entre consumo de etanol y BEL o distancia recorrida en el OF fueron analizadas vía Pearson. El valor eta-cuadrado parcial (η^2p) fue usado para informar del tamaño del efecto de los ANOVAs. El test de Tukey fue usado para explorar los efectos significativos principales e interacciones arrojadas por los ANOVAs.

En todos los casos, se consideraron diferencias estadísticamente significativas cuando el valor de $p \leq 0,05$.

Resultados y discusión

Experimento 1 – Modelo "From BE to BD"

En cuanto al consumo de agua una vez adquirido el consumo de los DPP tipo BE (ver Figura 10), se encontró que las ratas expuestas a BE y a dos botellas de agua (0% etanol) consumían significativamente más agua que el resto de los grupos (excepto por aquellas expuestas a BE y una solución de etanol al 14%).

Con relación al consumo neto de etanol (ver Figura 11), las ratas que fueron expuestas a BE y a una solución de etanol del 2%, 6% o 10% consumieron significativamente más etanol que sus pares expuestos a "control eating condition", alcanzando consumos de hasta 6,3 g/kg de etanol. De entre las ratas expuestas a BE, las ratas en el grupo de solución de etanol al 10% consumieron significativamente más alcohol que sus pares del resto de grupos.

Los valores de preferencia por la botella con contenido alcohólico (ver Figura 12) se mantuvieron de media altos para todos los grupos (entorno al 80%), excepto para

aquellas ratas expuestas a BE y a la solución de etanol al 14%, hecho que puede ser debido a la reluctancia natural que exhiben las ratas por las soluciones de etanol a altas concentraciones, las cuales producen irritación gástrica (Kiefer & Dopp, 1989).

Los resultados previamente descritos muestran cómo la interacción del BE y BD en nuestro modelo produce una notable autoadministración por parte de las ratas, alcanzándose valores que rondan los 5 g/kg en 90 minutos de consumo y unos valores de preferencia por la solución de etanol del 80 – 90%, valores que sobrepasan los alcanzados en otros modelos de consumo voluntario (Carnicella et al., 2014; Colombo et al., 2014). Estos altos valores de consumo en tan poco tiempo se ajustan perfectamente tanto a la definición canónica de BE como al comportamiento de BD en humanos, demostrando de esta forma la validez del modelo.

Cuando no se provee a las ratas de una solución de etanol (o se provee una de elevada concentración y por lo tanto excesivamente aversiva), el BE ejerce un efecto facilitador en el consumo de agua (ver Figura suplementaria 1, panel superior), sin embargo, cuando se da la opción a consumir una solución de etanol con una concentración del 2 – 10% (ver Figura suplementaria 1, paneles centrales), las ratas expuestas a BE exhiben un consumo de solución de etanol notablemente más alto que sus pares expuestas a 6DPP, siendo el consumo de la botella de agua prácticamente despreciable (como muestran los valores de preferencia). Estos datos demuestran que la experiencia BE produce un efecto facilitador selectivo sobre el consumo de etanol bajo las condiciones ensayadas.

Los valores de consumo correlacionaron positiva y significativamente tanto con la distancia recorrida en el OF como con los BEL (representado en las Figuras 13 y 14), demostrándose de esta forma consecuencias comportamentales y fisiológicas tras el consumo de solución de etanol.

El consumo en el presente modelo está sujeto a varias interpretaciones. Es posible que la gran cantidad de azúcares en los DPP influyan en el patrón de consumo exhibido en nuestro modelo. Varios estudios muestran como la ingestión de alimentos con alto valor hedónico aumentan el posterior consumo de etanol en roedores (Avena et al., 2004; Blanco-Gandía et al., 2018). Además, la vía neural para el sabor dulce parece facilitar el

consumo de alcohol (Blednov et al., 2008; Lemon et al., 2004), lo cual puede tener un papel significativo en nuestro modelo.

Por otro lado, el BE produce la activación de regiones cerebrales similares que las drogas de abuso (Blum et al., 2017; Volkow et al., 2011). Es posible que BE estimule la vía de recompensa del cerebro y, debido a tal estimulación, promueva el consumo posterior de etanol. Futuras investigaciones deberían incluir la medición neurobiológica con el fin de corroborar esta teoría.

Experimento 2 – Modelo "From BE to BD": consumo a pesar de consecuencias aversivas

El ANOVA para el consumo neto de etanol durante las sesiones 11 – 15 arrojó un efecto significativo de la interacción de las variables "Group" x "Session". Como confirmaron los análisis post-hoc, sólo la adición de 0,3 g/L de quinina a la solución al 10% de etanol produjo una reducción significativa en el consumo, no encontrándose dicha reducción para el resto de las concentraciones (ver Figura 15).

El hecho de que se mantengan los niveles de consumo incluso cuando son adulterados con quinina a 0,1 g/L prueba la validez del modelo, siendo equiparable al consumo que se da en humanos con dependencia al alcohol, el cual se produce a pesar de tener la bebida un sabor aversivo (Leon et al., 2007).

Experimento 3 – Modelo "From BE to BD": evaluando la validez predictiva

De acuerdo con nuestras hipótesis iniciales, el tratamiento agudo con naltrexona en la sesión 12 de procedimiento produjo una reducción significativa del consumo de etanol (ver Figura 16) para aquellas ratas administradas con 10 mg/kg en comparación con aquellas administradas con vehículo o 1 mg/kg.

Puesto que el consumo de etanol se reduce tras la administración de un antagonista opioide, podemos asumir que dicho consumo está, al menos en parte, mediado por el sistema opioide. Este hecho, sumado a la efectividad sobre el modelo de un fármaco

aprobado para el tratamiento en humanos (Guardia Serecigni, 2015), demuestra la validez predictiva y de constructo del presente modelo.

Experimento 4 – Modelo "From BE to BD": modulación del S1-R

En cuanto a la administración aguda de antagonistas para S1-R en la sesión 12 del procedimiento (ver Figura 2), sorprendentemente ninguno de los tratamientos administrados (BD-1063 a 2, 8 o 32 mg/kg, o S1RA a 4, 6 o 64 mg/kg) resultaron eficaces para modificar el consumo voluntario de etanol en nuestro modelo. Tampoco se encontraron diferencias significativas respecto al control cuando las ratas fueron administradas diariamente con S1RA a 64 mg/kg (ver Figura 18).

Pese a que gran cantidad de estudios muestran la eficacia en la reducción del consumo de etanol del antagonismo para S1-R (principalmente del tratamiento con BD-1063) (Quadir et al., 2019), existen otros trabajos que informan de la falta del efecto de estos cuando se administran a ratas Wistar no dependientes (Sabino et al., 2009).

De igual forma, tampoco se obtuvieron cambios en el consumo con el tratamiento agudo con el agonista PRE-084 a cualquiera de las dosis ensayadas (ver Figura 17), hecho que puede estar relacionado con que la administración del fármaco se realizase una vez adquirido el comportamiento de consumo. Futuros experimentos deben ensayar el posible efecto facilitador sobre el consumo si se administra un agonista S1-R de forma preventiva al procedimiento o en las primeras sesiones de este (cuando la conducta de consumo aún no se ha establecido).

Experimento 5 – BD en adolescentes: efecto del antagonismo S1-R

Con relación al experimento diseñado para evaluar la eficacia del antagonismo para S1-R en reducir el consumo en forma de BD de ratas macho y hembras adolescentes, el tratamiento con S1RA (Experimento 5A, ver Figura 19) resultó efectivo en todas las sesiones cuando fue administrado a 64 mg/kg tanto para machos como para hembras. El tratamiento con 16 mg/kg resultó en una reducción significativa en el consumo de etanol de las hembras en las sesiones 2 – 6 y de los machos en las sesiones 1 y 2. La dosis más baja administrada de S1RA (4 mg/kg) produjo una reducción significativa en el consumo

de las hembras en las sesiones 2 – 5 y en el consumo de los machos en la sesión 3. En cuanto al tratamiento con BD-1063 (Experimento 5B, ver figura 20), las ratas tratadas con 32 mg/kg mostraron una reducción significativa en el consumo con independencia del sexo o la sesión.

Por lo tanto, ambos tratamientos resultaron efectivos (especialmente el tratamiento con S1RA a 64 mg/kg) en la reducción del consumo de etanol en el modelo de consumo en adolescencia tanto en machos como en hembras. A nuestro saber, es la primera vez que se informa de dicho efecto en este tramo de edad y en hembras en general.

Los valores de consumo en el post-test (representados en la Figura 21) fueron inferiores tanto para hembras que fueron tratadas con 4, 16 o 64 mg/kg de S1RA como para machos que fueron tratados con 16 o 64 mg/kg de S1RA en comparación con sus respectivos controles. En cuanto a aquellos animales que fueron tratados con BD-1063, y guiados por nuestra hipótesis a priori, se realizaron comparaciones planeadas entre los grupos de 0 y 32 mg/kg, encontrando un menor consumo para este último. No se encontraron efectos principales ni interacciones significativas en el test NOR.

El efecto persistente encontrado en la reducción del consumo, el cual sucede mucho después de que los fármacos hayan sido metabolizados (Gris et al., 2016), puede estar debido a una alteración de los efectos a largo plazo de la exposición crónica al etanol. Este fenómeno también ha sido informado para el tratamiento con naloxona (Salguero et al., 2020) y en estudios en los que antagonistas para S1-R han bloqueado la sensibilización conductual inducida por cocaína (Ujike et al., 1996).

Conclusiones

1. El procedimiento llevado a cabo en el modelo “From binge eating to binge drinking” (BE-BD) conduce a un notable consumo voluntario de etanol en ratas Wistar macho adultas. Puesto que el modelo no requiere una habituación o preexposición extensiva al alcohol, permite investigar todas las fases de la adicción al mismo.

2. Una vez establecido el consumo, tanto la ingesta de etanol como de DPP en el modelo “BE-BD” mostraron ser resistentes a la adulteración con quinina y las ratas exhibieron signos de intoxicación. Por lo tanto, el modelo demostró validez aparente y de constructo en tanto en cuanto el consumo voluntario ocurría a pesar de consecuencias aversivas, se daba de forma rápida y producía consecuencias comportamentales y fisiológicas.

3. El consumo de etanol en el modelo “BE-BD” es sensible al tratamiento con naltrexona, demostrando por tanto la validez predictiva del modelo y que dicho consumo está mediado, al menos en parte, por el sistema opioide.

4. La administración sistémica de ligandos S1-R en el modelo “BE-BD” no produjo ninguna modulación en el consumo de etanol. Ni los antagonistas S1RA y BD-1063 ni el agonista PRE-084 ejercieron acción alguna. Se deben realizar más investigaciones para dilucidar esta discrepancia con estudios previos sobre la eficacia de la ingesta de etanol de fármacos para esta diana terapéutica.

5. El antagonismo S1-R inducido por la administración subcutánea de S1RA y BD-1063 bloqueó el consumo de etanol en un modelo de autoadministración de etanol tipo BD en ratas Wistar adolescentes macho y hembra, lo que además redujo la ingesta de etanol de libre elección mucho después de la eliminación de dichos compuestos. Esta es la primera evidencia de la efectividad del antagonismo S1-R sobre el consumo de etanol en adolescentes y hembras.

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Abbreviations

2-BC	Two-bottle choice paradigm
5-HT	Serotonin
5-HT$_3$R	5-Hydroxytryptamine Receptors Subtype 3
ADHs	Alcohol dehydrogenases
AMG	Amigdala
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AUD	Alcohol use disorder
BD	Binge drinking
BD-1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride
BE	Binge eating
BEL	Blood ethanol level
BK	Large-conductance Ca^{2+}-activated K^+ channel
CB$_1$R	Cannabinoid receptor tipe 1
CB$_2$R	Cannabinoid receptor tipe 2
CCK	Cholecystokinin
ChI	Cholinergic interneurons
CNS	Central nervous system
CPP	Conditioned place preference
CRH	Corticotropin-releasing hormone
DA	Dopamine
DALYs	Deaths and disability-adjusted life-years
DOR	δ-opioid receptor
DPP	Dustless precision pellets
DRN	Dorsal raphe nuclei
DYN	Dynorphin
EMA	European Medicines Agency
FDA	U.S Food and Drug Administration
FID	Flame ionization detector
GABA	Gamma-Aminobutyric acid
GABA$_A$R	Gamma-Aminobutyric acid receptor type A
GIRK	G-protein-coupled inwardly rectifying K^+ channel
Glu	Glutamate
GlyRs	Glycine receptors
HED	Heavy episodic drinking
HPC	Hippocampus
KOR	κ-opioid receptor
LPT	Long term potentiation
LTD	Long term depression
MOR	μ-opioid receptor
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neuron
Nac	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NOR	Novel object recognition test
OF	Open field test
PD	Postnatal day
PEG	Polyethylene glycol
PFC	Prefrontal cortex
PRE-084	2-(4-morpholinoethyl)-1-phenylcyclohexane-1-carboxylate Hydrochloride
S1-R	Sigma-1 receptor
S1RA	4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride
SERT	Serotonin reuptake transporter
SIP	Scheduled-induced polydipsia
SN	Substantia nigra
sNP	Sardinian alcohol non-preferring rat
sP	Sardinian alcohol-preferring rat
STR	Striatum
UChA	Low alcohol-drinking rat
UChB	High-alcohol drinking rat
VTA	Ventral tegmental area
Δ^9-THC	Delta-9-tetrahydrocannabinol

List of publications

Articles directly related to this Doctoral Thesis.

- **Ruiz-Leyva, L.**, Salguero, A., Morón, I., Portillo-Salido, E., Cendán, C. M., & Pautassi, R. M. (2020). Sigma-1 antagonism inhibits binge ethanol drinking at adolescence. Drug and Alcohol Dependence, 215, 108214.
  Impact factor: 2020: 4.492. **Quartile:** Q1 (Substance Abuse) 3/21.

- **Ruiz-Leyva, L.**, Vázquez-Ágredos, A., Jiménez-García, A. M., López-Guarnido, O., Pla, A., Pautassi, R. M., ... & Cendán, C. M. (2022). From binge eating to binge drinking: A new and robust paradigm for assessing binge ethanol self-administration in male rats. Addiction Biology, 27(2), e13153.
- **Impact factor:** 2021: 4.093. **Quartile:** Q3 (Substance Abuse) 11/21.

Other articles

- Jiménez-García, A. M., **Ruíz-Leyva, L.**, Cendán, C. M., Torres, C., Papini, M. R., & Morón, I. (2016). Hypoalgesia induced by reward devaluation in rats. PloS one, 11(10), e0164331.

- Jiménez-García, A. M.*, **Ruiz-Leyva, L.***, Vázquez-Ágredos, A., Torres, C., Papini, M. R., Cendán, C. M. and Morón, I. (2019). Consummatory Successive Negative Contrast in Rats. Bio-protocol 9(7): e3201.

- Salguero, A., Suarez, A., Luque, M., **Ruiz-Leyva, L.**, Cendán, C. M., Morón, I., & Pautassi, R. M. (2020). Binge-Like, Naloxone-Sensitive, Voluntary Ethanol Intake at Adolescence Is Greater Than at Adulthood, but Does Not Exacerbate Subsequent Two-Bottle Choice Drinking. Frontiers in Behavioral Neuroscience, 14.

- Perea, C.*, Vázquez-Ágredos, A.*, **Ruiz-Leyva, L.***, Morón, I., Zúñiga, J. M., & Cendán, C. M. (2021). Caloric Restriction in Group-Housed Mice: Littermate and Sex Influence on Behavioral and Hormonal Data. Frontiers in Veterinary Science, 8, 349.

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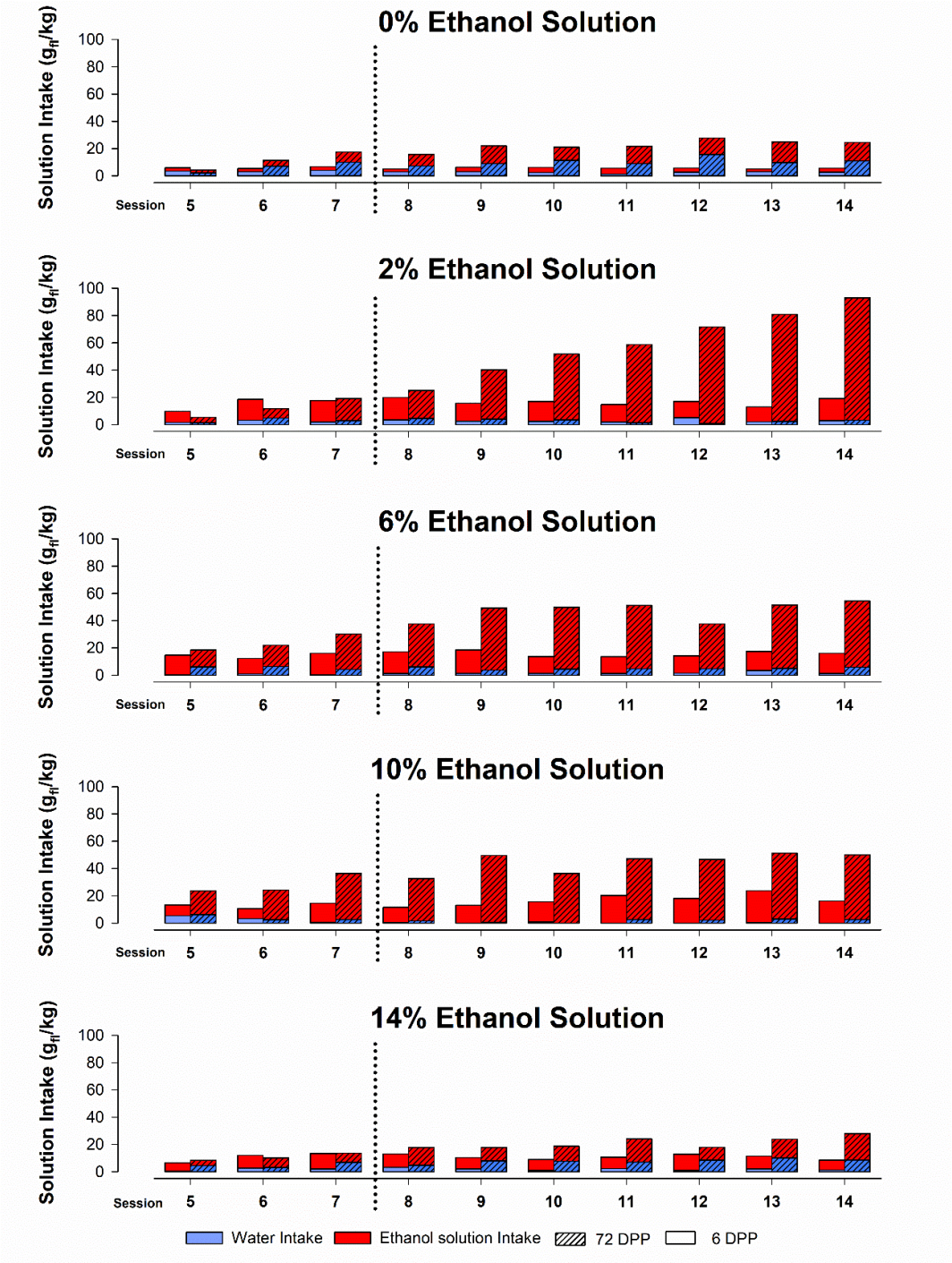
Annexes



Supplementary figure 1. Overall fluid intake (g_{fl}/kg) across sessions. Each stacked bar represents the mean overall fluid intake in each session, with the blue and red section of each bar representing the amount of water and ethanol drank, respectively. Please note that, for the 0% ethanol condition, the red section of the bar indicates intake from one of the two bottles filled with water (i.e., a solution with 0% of ethanol content) that was randomly marked as "experimental". Each group was composed by 6 – 13 rats. The solid bars present the scores of the control groups given 6 DPP, whereas the bars filled with oblique stripes present the scores of the BE groups (i.e., those exposed to 72 DPP). The vertical dotted line helps differentiate the Acquisition phase (sessions 5 – 7) from the Binge Eating consumption phase (sessions 8 – 14).

Supplementary Table 1

Experiment 1

		Males				Females	
	S1RA dose	0 mg	4 mg	16 mg	64 mg	0 mg	4
Intake habituation session	Ethanol intake (g/kg)	3.92 ± 0.61	4.24 ± 1.15	1.99 ± 0.29	3.49 ± 1.09	5.41 ± 2.3	2
	Ethanol intake (%)	19.83 ± 1.49	22.42 ± 5.12	12.65 ± 1.68	22.27 ± 7.76	23.43 ± 8.82	1
	Overall fluid intake (ml/100 g of body weight)	24.92 ± 1.38	25.89 ± 2.77	22.5 ± 0.97	21.76 ± 1.67	25.19 ± 2.2	1
Post-test	Ethanol intake (g/kg)	4.34 ± 0.82	4.92 ± 0.69	1.57 ± 0.52	1.08 ± 0.18	4.83 ± 1.14	1
	Ethanol intake (%)	27.49 ± 5.43	44.76 ± 6.74	16.58 ± 6.08	9.68 ± 1.88	35.34 ± 6.81	1
	Overall fluid intake (ml/100 g of body weight)	59.09 ± 6.24	42.17 ± 2.22	35.91 ± 2.18	44.10 ± 1.79	34.8 ± 2.6	2
Experiment 2							
	BD-1063 dose	0 mg	2 mg	8 mg	32 mg	0 mg	2
Intake habituation session	Ethanol intake (g/kg)	1.76 ± 0.2	2.39 ± 0.34	1.82 ± 0.18	1.45 ± 0.11	2.71 ± 0.44	2
	Ethanol intake (%)	10.79 ± 1.79	13.42 ± 1.88	10.78 ± 1.22	8.62 ± 0.9	16.36 ± 2.34	1
	Overall fluid intake (ml/100 g of body weight)	21.49 ± 1.09	22.57 ± 1.11	21.37 ± 0.62	21.53 ± 0.97	20.33 ± 0.95	2
Post-test	Ethanol intake (g/kg)	3.94 ± 1.21	2.81 ± 1.29	3.27 ± 1.36	0.85 ± 0.21	2.25 ± 0.59	2
	Ethanol intake (%)	36.6 ± 8.04	24.91 ± 9.64	31.34±12.12	6.78 ± 1.31	25.15 ± 4.70	2
	Overall fluid intake (ml/100 g of body weight)	15.50 ± 0.73	14.54 ± 0.58	14.69 ± 0.56	16.48 ± 0.66	15.07 ± 0.98	1
NOR Habituation	Distance (cm)	3880.06 ±221.57	4547.2 ±287.85	4387.6 ±404.89	3787.4 ±277.73	3193.98 ±238.82	3 ±
NOR Familiarization	Total exploration time (s)	66.65 ± 7.81	54.59 ± 6.10	64.22 ± 4.39	57.03 ± 7.07	64.83 ± 8.71	6
NOR Test	Discrimination Index (Di)	0.18 ± 0.07	0.22 ± 0.1	0.21 ± 0.08	0.29 ±0.07	0.25 ±0.11	0 ±

Supplementary table 1: Intake scores at the habituation and post-test (i.e., post-binge) sessions (Experiments 1 and 2) and time and discriminative scores at the Novel Object Recognition test (NOR, Experiment 2). The NOR also included rats nev (i.e., naïve). Scores for these rats are not shown.